

66-

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> : <b>C12N 15/13, C07K 15/28, A61K 39/395, G01N 33/577</b>		<b>A2</b>	(11) International Publication Number: <b>WO 94/29451</b>	
			(43) International Publication Date: 22 December 1994 (22.12.94)	
(21) International Application Number: <b>PCT/GB94/01291</b>		(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).		
(22) International Filing Date: 15 June 1994 (15.06.94)		<b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>		
(30) Priority Data:				
9312415.4	16 June 1993 (16.06.93)			GB
9401597.1	27 January 1994 (27.01.94)			GB
9402499.9	9 February 1994 (09.02.94)	GB		
9406222.1	29 March 1994 (29.03.94)	GB		
(71) Applicant (for all designated States except US): <b>CELLTECH LIMITED [GB/GB]; 216 Bath Road, Slough, Berkshire SL1 4EN (GB).</b>				
(72) Inventors; and				
(75) Inventors/Applicants (for US only): <b>MORGAN, Susan, Adrienne [GB/GB]; 1A Ennerdale Crescent, Slough, Berkshire SL1 6EH (GB). EMTAGE, John, Spencer [GB/GB]; 49, Temple Mill Island, Temple, Marlow, Buckinghamshire SL7 1SQ (GB). BODMER, Mark, William [GB/GB]; Rose Cottage, 5, Manor Road, South Hinksey, Oxford OX1 5AS (GB). ATHWAL, Diljeet, Singh [GB/GB]; 33 Casella Road, New Cross Gate, London SE17 (GB).</b>				
(74) Agent: <b>MERCER, Christopher, Paul; Carpmaels &amp; Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).</b>				
(54) Title: <b>HUMANISED ANTIBODIES</b>				
(57) Abstract				
<p>The invention describes humanised antibodies having specificity for the epitope recognised by the murine monoclonal antibody L243. Also described are processes for preparing said antibodies and pharmaceutical compositions and medical uses of said antibodies.</p>				

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LJ	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

## **HUMANISED ANTIBODIES**

### **FIELD OF THE INVENTION**

- 5 This invention relates to humanised antibodies, having specificity for the epitope recognised by the murine monoclonal antibody L243, to processes for preparing said antibodies, to pharmaceutical compositions containing said antibodies, and to medical uses of said antibodies.
- 10 The term humanised antibody molecule is used to describe a molecule having an antigen binding site derived from an immunoglobulin from a non-human species, the remaining immunoglobulin-derived parts of the molecule being derived from a human immunoglobulin. The antigen binding site may comprise either complete variable regions fused onto
- 15 human constant domains or only the complementarity determining regions (CDRs) grafted onto appropriate human framework regions in the variable domains.

### **BACKGROUND OF THE INVENTION**

- 20 The proteins encoded in the Major Histocompatibility Complex region of the genome are involved in many aspects of immunological recognition. It is known that all mammals and probably all vertebrates possess basically equivalent MHC systems and that immune response genes are linked to the MHC.
- 25 In man the major histocompatibility complex is the HLA gene cluster on chromosome 6. The main regions are D, B, C, and A. The D region contains genes for Class II proteins which are involved in cooperation and interaction between cells of the immune system. Many diseases have
- 30 been found to be associated with the D region of the HLA gene cluster. Studies to date have shown associations with an enormous variety of diseases including most autoimmune diseases (see for example European Patent No. 68790). European Patent No. 68790 suggests controlling diseases associated with a particular allele of certain regions of the MHC
- 35 such as the HLA-D region in humans by selectively suppressing the immune responses controlled by a monoclonal antibody specific for an MHC-Class II antigen.

L243 is a murine IgG2A anti-HLA DR antibody which we believe to be of particular use in treatment of diseases such as autoimmune diseases since it shows particularly potent suppression of *in vitro* immune function and is monomorphic for all HLA-DR.

Since most available monoclonal antibodies are of rodent origin, they are naturally antigenic in humans and thus can give rise to an undesirable immune response termed the HAMA (Human Anti-Mouse Antibody) response. Therefore, the use of rodent monoclonal antibodies as therapeutic agents in humans is inherently limited by the fact that the human subject will mount an immunological response to the antibody and will either remove it entirely or at least reduce its effectiveness.

Proposals have been made for making non-human MABs less antigenic in humans. Such techniques can be generically termed 'humanisation' techniques. These techniques generally involve the use of recombinant DNA technology to manipulate DNA sequences encoding the polypeptide chains of the antibody molecule. A simple form of humanisation involves the replacement of the constant regions of the murine antibody with those from a human antibody [Morrison *et al* (1984) Proc. Natl. Acad. Sci. USA **81** 6851-55; Whittle *et al* (1987) Prot. Eng. **1** 499-505]. The lowering of the level of the HAMA response to the chimeric antibodies leads to the expectation that further humanisation of the variable region outside of the antigen binding site may abolish the response to these regions and further reduce any adverse response.

A more complex form of humanisation of an antibody involves the redesign of the variable region domain so that the amino acids constituting the murine antibody binding site are integrated into the framework of a human antibody variable region. Humanisation has led to the reconstitution of full antigen binding activity in a number of cases [Co *et al* (1990) J. Immunol. **148** 1149-1154; Co *et al* (1992) Proc. Natl. Acad. Sci. USA **88** 2869-2873; Carter *et al* (1992) Proc. Natl. Acad. Sci. **89** 4285-4289; Routledge *et al* (1991) Eur. J. Immunol. **21** 2717-2725 and

International Patent Specifications Nos. WO 91/09967; WO 91/09968 and WO 92/11383].

5 It can therefore be anticipated that the humanisation of L243 may lead to reduced immunogenicity in man and overcome the potential problem of the HAMA response previously associated with the use of murine antibodies in humans.

10 We have now prepared recombinant antibody molecules having specificity for the epitope recognised by the murine monoclonal antibody L243.

### **SUMMARY OF THE INVENTION**

15 Thus according to a first aspect the invention provides a recombinant antibody molecule having specificity for antigenic determinants dependent on the DR $\alpha$  chain.

20 The term recombinant antibody molecule is used to denote an antibody produced using recombinant DNA techniques. The antibody is preferably a humanised antibody, e.g. a chimeric or CDR-grafted antibody.

In a preferred embodiment of the first aspect the invention provides a recombinant antibody molecule have specificity for the epitope recognised by the murine monoclonal antibody L243.

25 In a preferred embodiment of the first aspect of the present invention there is provided a humanised antibody molecule having specificity for the epitope recognised by the murine monoclonal antibody L243 and having an antigen binding site wherein at least one of the complementarity determining regions (CDRs) of the variable domain is derived from the  
30 mouse monoclonal antibody L243 (MAb L243) and the remaining immunoglobulin-derived parts of the humanised antibody molecule are derived from a human immunoglobulin or an analogue thereof, said humanised antibody molecule being optionally conjugated to an effector or reporter molecule.

35

The humanised antibody molecule may comprise a chimeric humanised antibody or a CDR-grafted humanised antibody. When the humanised antibody molecule comprises a CDR-grafted humanised antibody, the heavy and/or light chain variable domains may comprise only one or two  
5 MAb L243 derived CDRs; though preferably all three heavy and light chain CDRs are derived from MAb L243.

As described above L243 is a monoclonal antibody previously described by Lampson & Levy [J. Immunol. (1980) 125 293]. The amino acid  
10 sequences of the light and heavy chain variable regions of the antibody are shown in Figures 1 and 2 hereinafter. L243 has been deposited at the American Type Culture Collection, Rockville, Maryland USA under Accession number ATCC HB55.

15 **DETAILED DESCRIPTION OF THE INVENTION**

The humanised antibody of the present invention may have attached to it an effector or reporter molecule. For instance a macrocycle for chelating a heavy metal atom, or a toxin such as ricin, may be attached to the humanised antibody by a covalent bridging structure. Alternatively, the  
20 procedure of recombinant DNA technology may be used to produce a humanised antibody molecule in which the Fc fragment, C<sub>H</sub>3 or C<sub>H</sub>2 domain of a complete antibody molecule has been replaced by or has attached thereto by peptide linkage a functional non-immunoglobulin protein such as an enzyme or toxin molecule.

25 The humanised antibody of the present invention may comprise a complete antibody molecule, having full length heavy and light chains; a fragment thereof, such as a Fab, Fab', (Fab')<sub>2</sub>, or Fv fragment; a single chain antibody fragment, e.g. a single chain Fv, a light chain or heavy  
30 chain monomer or dimer; multivalent monospecific antigen binding proteins comprising two, three, four or more antibodies or fragments thereof bound to each other by a connecting structure; or a fragment or analogue of any of these or any other molecule with the same specificity as MAb L243.

35

In a preferred embodiment the antibody comprises a complete antibody molecule, having full length heavy and light chains.

5 The remaining non-L243 immunoglobulin derived parts of the humanised antibody molecule may be derived from any suitable human immunoglobulin. For instance where the humanised antibody molecule is a CDR-grafted humanised antibody molecule, appropriate variable region framework sequences may be used having regard to class/type of the donor antibody from which the antigen binding regions are derived.  
10 Preferably the type of human framework used is of the same/similar class/type as the donor antibody. Advantageously the framework is chosen to maximise/optimize homology with the donor antibody sequence particularly at positions spacially close or adjacent to the CDRs. Examples of human frameworks which may be used to construct CDR-grafted antibodies are LAY, POM, TUR, TEI, KOL, NEWM, REI and EU;  
15 for instance KOL and NEWM for the heavy chain and REI for the light chain or EU for both the heavy chain and light chain.

20 An alternative procedure for the selection of a suitable human framework involves aligning the framework regions of the light chain of the non-human framework with those of the four human light chain subgroups identified by Kabat *et al* (1991) [in: Sequences of Proteins of Immunological Interest, Fifth Edition]. The consensus sequence for the light chain subgroup most homologous to the non-human antibody light  
25 chain is chosen.

Any differences between the framework residues of the non-human antibody and the consensus human group light chain sequence are analysed for the potential contribution they may have to antigen binding as  
30 described in Published International Patent Application No. WO91/09967. Based on this analysis some or all of the residues identified may be altered. The same procedure is carried out for the selection of a suitable framework to accept the non-human heavy chain CDRs.

35 For constructing the L243 CDR grafted light chain, the human subgroup 1 consensus sequenc was found to be particularly suitable, and for



constructing the L243 grafted heavy chain the human subgroup 1 consensus sequence was also found to be particularly suitable.

5 The light or heavy chain variable domains of the humanised antibody molecule may be fused to human light or heavy chain constant domains as appropriate, (the term 'heavy chain constant domains' as used herein are to be understood to include hinge regions unless specified otherwise). The human constant domains of the humanised antibody molecule, where present, may be selected having regard to the proposed function of the antibody, in particular the lack of effector functions which may be required. 10 For example, the heavy chain constant domains fused to the heavy chain variable region may be human IgA, IgG or IgM domains. Preferably human IgG domains are used. Light chain human constant domains which may be fused to the light chain variable region include human 15 Lambda or human Kappa chains.

Analogues of human constant domains may alternatively be advantageously used. These include those constant domains containing one or more additional amino acids than the corresponding human domain or those constant domains wherein one or more existing amino acids of 20 the corresponding human domain has been deleted or altered. Such domains may be obtained, for example, by oligonucleotide directed mutagenesis.

25 The remainder of the humanised antibody molecule need not comprise only protein sequences from human immunoglobulins. For instance, a gene may be constructed in which a DNA sequence encoding part of a human immunoglobulin chain is fused to a DNA sequence encoding the amino acid sequence of a polypeptide effector or reporter molecule. 30

We have found that by modifying one or more residues in the N-terminal region of the C<sub>H</sub>2 domain of the L243 antibody we produce an antibody with an altered ability to fix complement as compared to unaltered antibody. 35

The amino acid residue which is altered preferably lies within amino acid positions 231 to 239, preferably within amino acid positions 234 and 239.

5 In a particularly preferred embodiment the amino acid residue which is altered is either Leu 235 and/or Gly 237.

10 As used herein the term 'altered' when used in conjunction with the ability of an antibody to fix complement most usually indicates a decrease in the ability of antibody to fix complement compared to the starting unaltered antibody. By choosing an appropriate amino acid to alter it is possible to produce an antibody the ability of which to fix complement is substantially reduced such as for example by altering residue Leu 235. It is also possible to produce an antibody with an intermediate ability to fix complement as compared to unaltered antibody by for example altering  
15 amino acid residue Gly 237.

20 As used herein the phrase 'substantially' reduce complement fixation denotes that human complement fixation is preferably  $\leq 30\%$ , more preferably  $\leq 20\%$  and is most preferably  $\leq 10\%$  of the level seen with wild type antibody.

25 Due to the alteration of one or more amino acid residues in the N-terminal region of the C<sub>H</sub>2 domain the antibody will preferably not bind significantly to FcRI and will bind to FcRIII receptor.

The residue numbering used herein is according to the Eu index described in Kabat *et al* [(1991) in: Sequences of Proteins of Immunological Interest, 5th Edition, United States Department of Health and Human Services].

30 The alterations at position 235 of replacing leucine by glutamic acid or alanine have been found particularly effective at producing a potent immunosuppressive L243 antibody with minimal toxicity *in vitro*.

35 The alteration at position 237 of replacing glycine by alanine has been found to produce an antibody with an intermediate ability to fix complement i.e. the complement fixation level is approximately 15-80%,

preferably 20-60% most preferably 20-40% of that seen with the wild type antibody.

5 The residue(s) could similarly be replaced using an analogous process to that described herein, by any other amino acid residue or amino acid derivative, having for example an inappropriate functionality on its side chain. This may be achieved by for example changing the charge and/or polarity of the side chain.

10 The term 'significantly' as used with respect to FcRI binding denotes that the binding of antibody to FcRI is typically  $\leq 20\%$ , and is most preferably  $\leq 10\%$  of that seen with unaltered antibody.

15 The standard techniques of molecular biology may be used to prepare DNA sequences coding for the antibodies according to the invention. Desired DNA sequences may be synthesised completely or in part using oligonucleotide synthesis techniques. Site-directed mutagenesis and polymerase chain reaction (PCR) techniques may be used as appropriate.

20 Suitable processes include the PCR strand overlap procedure and PCR mutagenesis as described in for example "PCR Technology Principles and Applications for DNA Amplification" (1989), Ed. H.A. Erlich, Stockholm Press, N.Y., London, and oligonucleotide directed mutagenesis [Kramer et al, Nucleic. Acid. Res. 12 9441 (1984)]. Suitable techniques are also  
25 disclosed in Published European Patent No. EP307434B.

The altered L243 with altered complement fixing ability may also be produced by for example, deleting residues such as 235, or by for example, inserting a glycosylation site at a suitable position in the  
30 molecule. Such techniques are well known in the art, see for example the teaching of published European patent application EP-307434.

The altered L243 may also be produced by exchanging lower hinge regions of antibodies of different isotypes. For example a G1/G2 lower  
35 hinge exchange abolished complement fixation.

The G1/G2 lower hinge exchange results in an antibody with altered residues in the 231-238 region of the N-terminal region of the C<sub>H</sub>2 domain, wherein one or more residues may be altered or deleted.

- 5 According to a second aspect of the invention there is provided a process for producing the humanised antibody of the first aspect of the invention which process comprises:
- 10 a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy or light chain comprising a variable domain wherein at least one of the CDRs of the variable domain is derived from the L243 MAb and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin;
- 15 b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light or heavy chain comprising a variable domain wherein at least one of the CDRs of the variable domain is derived from the MAb L243 and the remaining immunoglobulin-derived parts of the antibody chain are derived from
- 20 a human immunoglobulin;
- c) transfecting a host cell with both operons; and
- 25 d) culturing the transfected cell line to produce the humanised antibody molecule.

In a preferred embodiment of this aspect of the invention at least one of the expression vectors contains a DNA sequence encoding an antibody

30 heavy chain in which one or more amino acid residues the N-terminal region of the C<sub>H</sub>2 domain of said antibody has been altered from that in the corresponding unaltered antibody.

The alteration in the N-terminal region of the C<sub>H</sub>2 domain may be made

35 after the whole unmodified antibody has been expressed using techniques such as site directed mutagenesis.

The cell line may be transfected with two vectors, the first vector containing the operon encoding the light chain-derived polypeptide and the second vector containing the operon encoding the heavy chain derived polypeptide. Preferably the vectors are identical except in so far as the coding sequences and selectable markers are concerned so as to ensure as far as possible that each polypeptide chain is equally expressed.

Alternatively, a single vector may be used, the vector including the operons encoding both light chain- and heavy chain-derived polypeptides, and a selectable marker.

The alteration in the N- terminal region of the C<sub>H</sub>2 domain, e.g. at position 235 of the C<sub>H</sub>2 domain of the molecule may be introduced at any convenient stage in the humanisation e.g. CDR-grafting process. It is conveniently introduced after the variable domains have been grafted onto the heavy chains.

In further aspects, the invention also includes DNA sequences coding for the heavy and light chains of the antibodies of the present invention, cloning and expression vectors containing these DNA sequences, host cells transformed with these DNA sequences and processes for producing the heavy or light chains and antibody molecules comprising expressing these DNA sequences in a transformed host cell.

The general methods by which the vectors may be constructed, transfection methods and culture methods are well known *per se* [see for example Maniatis *et al* (1982) (Molecular Cloning, Cold Spring Harbor, New York) and Primrose and Old (1980) (Principles of Gene Manipulation, Blackwell, Oxford) and the examples hereinafter].

The DNA sequences which encode the L243 light and heavy chain variable domain amino acid sequences (and the corresponding deduced amino acid sequences) are given hereafter in Figures 1 and 2 respectively.

DNA coding for human immunoglobulin sequences may be obtained in any appropriate way. For example, amino acid sequences of preferred human acceptor frameworks such as, LAY, POM, KOL, REI, EU, TUR, TEI and NEWM are widely available to workers in the art. Similarly the  
5 consensus sequences for human light and heavy chain subgroups are available to workers in the art.

The standard techniques of molecular biology may be used to prepare DNA sequences coding for CDR-grafted products. Desired DNA  
10 sequences may be synthesised completely or in part using oligonucleotide synthesis techniques. Site-directed mutagenesis and polymerase chain reaction (PCR) techniques may be used as appropriate. For example, oligonucleotide directed synthesis [Jones *et al* (1986) Nature 321 522-525] and also oligonucleotide directed mutagenesis of a pre-existing variable  
15 domain region [Verhoeyen *et al* (1988) Science 239 1534-1536; Reichmann *et al* (1988) Nature 332 323-327].

Enzymatic filling-in of gapped oligonucleotides using T4 DNA polymerase [Queen *et al* (1989) Proc. Natl. Acad. Sci. USA 86 10029-10033;  
20 International Patent Application No. WO 90/07861] may be used.

Any suitable host cell/vector system may be used for expression of the DNA sequences coding for the chimeric or CDR-grafted heavy and light chains. Bacterial e.g. E.coli and other microbial systems may be used  
25 advantageously in particular for expression of antibody fragments, e.g. Fv, Fab and Fab' fragment and single chain antibody fragments e.g. single chain Fvs. Eucaryotic e.g. mammalian host cell expression systems may also be used to obtain antibodies according to the invention, particularly for production of larger chimeric or CDR-grafted antibody products.  
30 Suitable mammalian host cells include COS cells and CHO cells [Bebbington C R (1991) Methods 2 136-145] and myeloma or hybridoma cell lines, for example NSO cells [Bebbington C R *et al* (1992) Bio/Technology 10 169-175]. The use of CHO cells is especially preferred.

In the humanised antibody according to the invention, the heavy and light chain variable domains may comprise either the entire variable domains of MAb L243, or may comprise framework regions of a human variable domain having grafted thereon one, some or all of the CDRs of MAb L243.

5 Thus the humanised antibody may comprise a chimeric humanised antibody or a CDR-grafted humanised antibody.

When the humanised antibody is a CDR-grafted humanised antibody, in addition to the CDRs, specific variable region framework residues may be

10 altered to correspond to non-human i.e. L243 mouse residues. Preferably the CDR-grafted humanised antibodies of the present invention include CDR-grafted humanised antibodies as defined in our International Patent Specification No. WO-A-91/09967. The disclosure of WO-A-91/09967 is incorporated herein by reference.

15 Preferably the CDRs of the heavy chain correspond to the L243 residues at all of CDR1 (31 to 35), CDR2 (50 to 65) and CDR3 (95 to 102). Preferably the CDRs of the light chain correspond to L243 residues at all of CDR1 (24 to 34) CDR2 (50 to 56) and CDR3 (89 to 97). In addition the

20 heavy chain may have mouse L243 residues at one or more of residues 27, 67, 69, 71, 72 and 75. Similarly the light chain may have mouse L243 residues at one or more positions 45, 49, 70 and 71.

The invention further provides a CDR-grafted humanised antibody heavy

25 chain having a variable region domain comprising acceptor frameworks derived from human subgroup consensus sequence 1 and L243 donor antigen binding regions wherein the framework comprises L243 donor residues at one or more of positions 27, 67, 69, 71, 72 and 75.

30 The invention further provides a CDR-grafted humanised antibody light chain having a variable region domain comprising acceptor frameworks derived from human subgroup consensus sequence 1 and L243 donor antigen binding regions wherein the framework comprises L243 donor residues at one or more of positions 45, 49, 70 and 71.

35

The heavy chain may further have mouse L243 residues at one or more of residues 2, 9, 11, 16, 17, 20, 38, 43, 46, 80, 81, 82, 82a, 82b, 83, 84, 89, 91, 108 and 109.

- 5 The light chain may further have mouse L243 residues at one or more of residues 9, 13, 17, 18, 37, 40, 43, 45, 48, 49, 72, 74, 76, 80, 84, 85, 100, 103 and 104.

- 10 The antibody according to the invention may be a complete antibody or as explained above, a fragment thereof, a monomer or dimer or a multivalent monospecific antigen binding protein. Certain compounds of this latter group are particularly advantageous in that they possess high avidity. See for example Published International Patent Specification No. WO 92/01472 the teaching of which is incorporated herein.

- 15 Thus according to a further particular aspect of the invention we provide a multivalent monospecific antigen binding protein comprising two, three, four or more antibodies or fragments thereof bound to each other by a connecting structure which protein is not a natural immunoglobulin, each  
20 of said antibodies or fragments having a specificity for the epitope recognised by murine MAb L243 said antigen binding protein being optionally conjugated with an effector or reporter molecule.

- 25 In this aspect of the invention each antibody or fragment is preferably a humanised antibody or a fragment thereof as defined above and the multivalent monospecific antigen binding protein is thus a humanised multivalent monospecific antigen binding protein. Non-humanised e.g. murine, multivalent monospecific antigen binding proteins can, however, be contemplated and the invention is to be understood to also extend to  
30 these.

- The multivalent antigen binding protein, preferably comprises two, three or four antibodies or fragments thereof bound to each other by a connecting structure.



Immunological diseases which may be treated with the antibodies of the invention include for example joint diseases such as ankylosing spondylitis, juvenile rheumatoid arthritis, rheumatoid arthritis; neurological disease such as multiple sclerosis; pancreatic disease such as diabetes, juvenile onset diabetes; gastrointestinal tract disease such as chronic active hepatitis, celiac disease, ulcerative colitis, Crohns disease, pernicious anaemia; skin diseases such as psoriasis; allergic diseases such as asthma and in transplantation related conditions such as graft versus host disease and allograft rejection. Other diseases include those described in European Patent No. 68790.

The present invention also includes therapeutic and diagnostic compositions containing the antibodies of the invention. Such compositions typically comprise an antibody according to the invention together with a pharmaceutically acceptable excipient, diluent or carrier, e.g. for *in vivo* use.

Thus in a further aspect the invention provides a therapeutic, pharmaceutical or diagnostic composition comprising an antibody according to the invention, in combination with a pharmaceutically acceptable excipient, diluent or carrier.

The invention also provides a process for the preparation of a therapeutic, pharmaceutical or diagnostic composition comprising admixing an antibody according to the invention together with a pharmaceutically acceptable excipient, diluent or carrier.

The antibodies and compositions may be for administration in any appropriate form and amount according to the therapy in which they are employed.

The therapeutic, pharmaceutical or diagnostic composition may take any suitable form for administration, and, preferably is in a form suitable for parenteral administration e.g. by injection or infusion, for example by bolus injection or continuous infusion. Where the product is for injection or infusion, it may take the form of a suspension, solution or emulsion in an

oily or aqueous vehicle and it may contain formulatory agents such as suspending, preservative, stabilising and/or dispersing agents.

Alternatively, the antibody or composition may be in dry form, for reconstitution before use with an appropriate sterile liquid.

If the antibody or composition is suitable for oral administration the formulation may contain, in addition to the active ingredient, additives such as: starch e.g. potato, maize or wheat starch or cellulose or starch derivatives such as microcrystalline cellulose; silica; various sugars such as lactose; magnesium carbonate and/or calcium phosphate. It is desirable that, if the oral formulation is for administration it will be well tolerated by the patient's digestive system. To this end, it may be desirable to include in the formulation mucus formers and resins. It may also be desirable to improve tolerance by formulating the antibody or compositions in a capsule which is insoluble in the gastric juices. It may also be preferable to include the antibody or composition in a controlled release formulation.

If the antibody or composition is suitable for rectal administration the formulation may contain a binding and/or lubricating agent; for example polymeric glycols, gelatins, cocoa-butter or other vegetable waxes or fats.

Therapeutic and diagnostic uses typically comprise administering an effective amount of an antibody according to the invention to a human subject. The exact dose to be administered will vary according to the use of the antibody and on the age, sex and condition of the patient but may typically be varied from about 0.1mg to 1000mg for example from about 1mg to 500mg. The antibody may be administered as a single dose or in a continuous manner over a period of time. Doses may be repeated as appropriate.

The antibodies and compositions may be for administration in any appropriate form and amount according to the therapy in which they are employed. The dose at which the antibody is administered depends on the nature of the condition to be treated and on whether the antibody is

being used prophylactically or to treat an existing condition. The dose will also be selected according to the age and conditions of the patient. A therapeutic dose of the antibodies according to the invention may be, for example, between preferably 0.1-25mg/kg body weight per single  
 5 therapeutic dose and most preferably between 0.1-10 mg/kg body weight for single therapeutic dose.

The antibody may be formulated in accordance with conventional practice for administration by any suitable route and may generally be in a liquid  
 10 form (e.g. a solution of the antibody in a sterile physiologically acceptable buffer) for administration by for example an intravenous, intraperitoneal or intramuscular route.

The present invention is now described by way of example only, by  
 15 reference to the accompanying drawings in which:

- Figure 1: shows the nucleotide and amino acid sequence of L243  
 VI region
- Figure 2: shows the nucleotide and amino acid sequence of L243  
 20 Vh region
- Figure 3: shows a diagrammatic map of plasmid pMR15.1
- Figure 4: shows the nucleotide and amino acid sequence of VI  
 region in L243-gL1
- Figure 5: shows the nucleotide and amino acid sequence of VI  
 25 region of L243-gL2
- Figure 6: shows a diagrammatic map of plasmid pMR14.
- Figure 7: shows the nucleotide and amino acid sequence of Vh  
 region of L243-gH.
- Figure 8: shows a diagrammatic map of plasmid pGamma 1.
- 30 Figure 9: shows a graph of the results of a competition assay for  
 L243 grafts vs FITC-chimeric L243
- |        |        |
|--------|--------|
| —■—    | cH cL  |
| —+—    | cH gL1 |
| —*—    | gH cL  |
| 35 —□— | gH gL1 |

- Figure 10: shows a graph of a Scatchard analysis for L243 gamma 4
- cH cL Kd = 4.1nM
  - +— gH gL1 Kd = 6.4nM
  - \*— gH gL2 Kd = 9.6nM
- 5 Figure 11: shows a graph of FcRIII binding of chimeric and grafted L243 as measured by ADCC
- cH cL
  - \*— gH gL1
- Figure 12: shows a graph of L243 Isotype series MLR
- 10
- G1 L243 cH cL
  - +— G1 L243 gH gL1
  - ◇ Cyclosporin A
  - △ Medium control
  - ⊗ Responder alone
- Figure 13: shows a graph of L243 Isotype Series TT Response
- 15
- G1 L243 cH cL
  - +— G1 L243 gH gL1
  - ◇ Cyclosporin A
  - △ Medium control
  - ⊗ Responder alone
- 20
- Figure 14: shows the nucleotide and amino acid sequence of the hinge and part of the C<sub>H</sub>2 region of human C-gamma 1
- Figure 15: shows a graph of FcRIII binding of chimeric, grafted and grafted [L235E] L243 as measured by ADCC
- 25
- Chimeric G1 wt
  - +— Chimeric G1 [L235E]
  - \*— Graft G1 wt
  - Graft G1 [L235E]
- Figure 16: shows a graph of immunosuppressive activity of CDR grafted L243 measured by MLR
- 30
- Graft G1 wt
  - +— Graft G1 [L235E]
  - \* Cyclosporin A
  - X— Chimeric G1 wt
  - ◇ Chimeric G1 [L235E]
  - ⊗ Medium Control
- 35

- Figure 17: shows a graph of CDR graft d L243 and grafted [L235E] L243 TT recall response
- Graft G1 wt
  - +— Graft G1 [L235E]
  - \* Cyclosporin A
  - X— Chimeric G1 wt
  - ◇— Chimeric G1 [L235E]
  - X— Medium Control
- Figure 18: shows a graph of complement mediated cytotoxic potency of CDR grafted L243 and CDR grafted [L235E] L243
- Chimeric G1 wt
  - +— Chimeric G1 [L235E]
  - \* Graft G1 wt
  - Graft G1 [L235E]
- Figure 19: shows the nucleotide and amino acid sequences of
- a) Clone 43
  - b) Clone 183 and
  - c) Clone 192
- Figure 20: shows a diagrammatic map of plasmid pGamma 2.

## **DETAILED DESCRIPTION OF THE SPECIFIC EMBODIMENTS OF THE INVENTION**

25

### **EXAMPLE 1**

#### **Gene Cloning and Expression**

##### **RNA preparation from L243 hybridoma cells**

Total RNA was prepared from 3 x 10<sup>exp7</sup> L243 hybridoma cells as described below. Cells were washed in physiological saline and dissolved in RNAzol (0.2ml per 10<sup>exp6</sup> cells). Chloroform (0.2ml per 2ml homogenate) was added, the mixture shaken vigorously for 15 seconds and then left on ice for 15 minutes. The resulting aqueous and organic phases were separated by centrifugation for 15 minutes in an Eppendorf centrifuge and RNA precipitated from the aqueous phase by the addition of an equal volume of isopropanol. After 15 minutes on ice, the RNA was

pelleted by centrifugation, washed with 70% ethanol, dried and dissolved in sterile, RNAase free water. The yield of RNA was 350 µg.

Amino acid sequence of the L243 light chain.

- 5     The sequence of the first nine amino acids of the mature L243 light chain was determined to be NH<sub>2</sub>-DIQMTQSPAS.

PCR cloning of L243 Vh and Vl

- 10     The cDNA genes for the variable regions of L243 heavy and light chains were synthesised using reverse transcriptase to produce single stranded cDNA copies of the mRNA present in the total RNA, followed by Polymerase Chain Reaction (PCR) on the cDNAs with specific oligonucleotide primers.

15     a)     cDNA synthesis

- 20     cDNA was synthesised in a 20µl reaction containing the following reagents: 50mM Tris-HCl PH8.3, 75mM KCl, 10mM dithiothreitol, 3mM MgCl<sub>2</sub>, 0.5mM each deoxyribonucleoside triphosphates, 20 units RNAsin, 75ng random hexanucleotide primer, 2µg L243 RNA and 200 units Moloney Murine Leukemia Virus reverse transcriptase. After incubation at 42°C for 60 mins the reaction was terminated by heating at 95°C for 5 minutes.

25     b)     PCR

- 30     Aliquots of the cDNA were subjected to PCR using combinations of primers for the heavy and light chains. The nucleotide sequences of the 5' primers for the heavy and light chains are shown in Tables 1 and 2 respectively. These sequences, all of which contain a restriction site starting 6 nucleotides from their 5' ends, followed by the sequence GCCGCCACC to allow optimal translation of the resulting mRNAs, an initiator codon and a further 20 - 30 nucleotides, are a compilation based on the leader peptide sequences of known mouse antibodies [Kabat *et al* (1991) in Sequences of Proteins of Immunological Interest, 5th Edition -  
35     United States Department of Health and Human Services].

5 The 3' primers are shown in Table 3. The light chain primer spans the V - C junction of the antibody and contains a restriction site for the enzyme SphI to facilitate cloning of the VI PCR fragment. The heavy chain 3' primers are a mixture designed to span the J - C  
10 junction of the antibody. The first 23 nucleotides are identical to those found at the start of human C - gamma 1, 2, 3 and 4 genes and include the ApaI restriction site common to these human isotypes. The 3' region of the primers contain a mixed sequence based on those found in known mouse antibodies [Kabat E A, Wu, T.T.; Perry H M, Gottesman K S, and Foeller L; In: Sequences of Proteins of Immunological Interest, 5th Edition, US Department of Health and Human Services (1991)].

15 The combinations of primers described above enables the PCR products for Vh and VI to be cloned directly into the appropriate expression vector (see below) to produce chimeric (mouse - human) heavy and light chains and for these genes to be expressed in mammalian cells to produce chimeric antibodies of the desired isotype.

20 Incubations (20 µl) for the PCR were set up as follows. Each reaction contained 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01% w/v gelatin, 0.25 mM each deoxyribonucleoside triphosphate, 1 - 6 pmoles 5' primer mix (Table 4), 6 pmoles 3'  
25 primer, 1 µl cDNA and 0.25 units Taq polymerase. Reactions were incubated at 95°C for 5 minutes and then cycled through 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. After 30 cycles, aliquots of each reaction were analysed by electrophoresis on an agarose gel. Reactions containing 5' primer mixes B1, B2, B3 and  
30 B5 produced bands with sizes consistent with full length VI fragments while reaction B9 produced a fragment with a size expected of a Vh gene. The band produced by the B1 primers was not followed up as previous results had shown that this band corresponds to a light chain pseudogene produced by the  
35 hybridoma cell.

c) Molecular cloning of the PCR fragments.

5 DNA fragments produced in reactions B2, B3 and B5 were digested with the enzymes BstB1 and Spl1, concentrated by ethanol precipitation, electrophoresed on a 1.4 % agarose gel and DNA bands in the range of 400 base pairs recovered. These were cloned by ligation into the vector pMR15.1 (Figure 3) that had been restricted with BstB1 and Spl1. After ligation, mixtures were transformed into E. coli LM1035 and plasmids from the resulting bacterial colonies screened for inserts by digestion with BstB1 and Spl1. Representatives with inserts from each ligation were analysed further by nucleotide sequencing.

15 In a similar manner, the DNA fragments produced in reaction B9 and digested with HindIII and Apa1 were cloned into the vector pMR14 (Figure 6) that had been restricted with HindIII and Apa1. Again, representative plasmids containing inserts were analysed by nucleotide sequencing.

d) Nucleotide sequence analysis

20 Plasmid DNA (pE1701 and pE1702) from two isolates containing Vh inserts from reaction B9 was sequenced using the primers R1053 (which primes in the 3' region of the HCMV promoter in pMR14) and R720 (which primes in the 5' region of human C - gamma 4 and allows sequencing through the DNA insert on pMR14). The determined nucleotide sequence and predicted amino acid sequence of L243 Vh in pE1702 is given in Figure 2. The nucleotide sequence for the Vh insert in pE1701 was found to be identical to that in pE1702 except at nucleotide 20 (A in pE1701) and nucleotide 426 (A in pE1701). These two differences are in the signal peptide and J regions of Vh respectively and indicate that the two clones examined are independent isolates arising from the use of different primers from the mixture of oligonucleotides during the PCR stage.

35 To analyse the light chain clones, sequence derived from priming with R1053 was examined. The nucleotide sequence and predicted



amino acid sequence of the VI genes arising from reactions B2 (clone 183), B3 (clone 43) and B5 (clone 192) are shown in Figure 19. Comparison of the predicted protein sequences shows the following:

- 5
- i) clones 182, 183, 43 and 45 all code for a VI gene which, when the signal peptide is removed, produces a light chain whose sequence is identical to that determined by amino acid sequence analysis for L243 light chain (see above).
- 10
- ii) clones 182 and 183 contain a VI gene that codes for a signal peptide of 20 amino acids, while the VI gene in clones 43 and 45 results from priming with a different set of oligonucleotides and has a leader sequence of only 15 amino acids.
- 15
- iii) Clone 192 does not code for L243 VI. Instead, examination of the database of antibody sequences (Kabat, 1991) indicates that clone 192 contains the VI gene for MOPC21, a light chain synthesised by the NS1 myeloma fusion partner used in the production of the L243 hybridoma.
- 20
- iv) Clones 182 and 183 are identical except at nucleotide 26 (T in clone 182, C in clone 183). This difference can be accounted for by the use of different primers in the PCR and indicates that clones
- 25
- 182 and 183 are independent isolates of the same gene. The nucleotide sequence and predicted amino acid sequence of the complete VI gene from clone 183 is shown in Figure 1.

#### Construction of human gamma 1 and gamma 2 isotypes.

- 30 The L243 Vh gene was subcloned on a HindIII - ApaI fragment into pGamma 1 and pGamma 2, vectors containing the human C - gamma 1 and C - gamma 2 genes respectively (Figures 8 and 20).

#### Human isotype mutants

- 35 PCR mutagenesis was used to change residue 235 in human C - gamma1 contained in the vector pGamma 1 from leucine to either glutamic acid or

to alanine and to change residue 237 from glycine to alanine. The lower hinge region of human C-gamma 1 was also replaced by the corresponding region of human C-gamma 2. The following oligonucleotides were used to effect these changes:

5

## I) L235E change

R4911 5' GCACCTGAACTCGAGGGGGGACCGTCAGTC3'

R4910 5'CCCCCCTCGAGTTCAGGTGCTGAGGAAG3'

## 10 II) L235A change

R5081 5'GCACCTGAACTCGCAGGGGGGACCGTCAGTC3'

R5082 5'GACTGACGGTCCCCCTGCGAGTTCAGGTGC3'

## III) G237A change

15 R5088 5'GCACCTGAACTCCTGGGTGCACCGTCAGTC3'

R5087 5'GACTGACGGTGCACCCAGGAGTTCAGGTGC3'

## IV) Exchange of lower hinge regions

R4909 5'GCACCTCCAGTGGCAGGACCGTCAGTCTTCCTC3'

20 R4908 5'CGGTCCTGCCACTGGAGGTGCTGAGGAAGAG3'

Other oligonucleotides used in the PCR mutagenesis are:

R4732 5'CAGCTCGGACACCTTCTCTCCTCC3'

R4912 5'CCACCACCACGCATGTGACC3'

25

R4732 and R4912 prime between nucleotides 834 and 858 and between nucleotides 1156 and 1137 respectively in human C - gamma 1 (Figure 14).

30 The general strategy for the PCR mutagenesis was as follows. For each amino acid change, two rounds of PCR were used to generate DNA fragments containing the required substitutions. These fragments were then restricted with the enzymes Bgl II and StyI and used to replace the corresponding fragments containing the wild type sequence in the

35 pGamma 1 vector, (Figure 8).

For the first round PCR, reactions (20  $\mu$ l) were prepared containing the following reagents : 10 mM Tris - HCl pH 8.3, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01% gelatin, 0.25 mM each deoxyribonucleoside triphosphate, 50  $\mu$ g pGamma 1 DNA, 0.4 unit Taq polymerase and 6 pmoles of each of the  
5 primer. The following combinations of primers were used:

10 R4911 / R4912,  
R4910 / R4732,  
R5081 / R4912,  
R5082 / R4732,  
R5088 / R4912,  
R5087 / R4732,  
R4909 / R4912,  
R4908 / R4732.

15 After 30 cycles through 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, the reactions were extracted with chloroform, the newly synthesised DNA precipitated with ethanol, dissolved in water and electrophoresed on a 1.4 % agarose gel. Gel slices containing the DNA  
20 fragments were excised from the gel, the DNA recovered from the agarose using a "Mermaid™" kit (from Stratech Scientific Ltd., Luton, England) and eluted into 20  $\mu$ l sterile water.

25 Second round PCR was in a 100  $\mu$ l reaction containing 10 mM Tris - HCl pH 8.3, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01 % gelatin, 0.25 mM each deoxyribonucleoside triphosphate, 2 units Taq polymerase, 1/20 of each pair of DNA fragments from the first round reaction and 30 pmoles of each of R4732 and R4912. After 30 cycles, see above, the reactions were extracted with phenol / chloroform (1/1) and precipitated with ethanol.  
30 Fragments were digested with Bgl11 and Sty1, electrophoresed on a 1.4 % agarose gel and DNA bands of 250 base-pairs recovered from gel slices as previously described.

35 These Bgl II - Sty1 fragments were ligated in a 3 - way ligation to the 830 base-pair Sty1 - EcoR1 fragment, containing the C - terminal part of the C<sub>H</sub>2 domain and the entire C<sub>H</sub>3 domain of human C - gamma 1, and the

BglII - EcoRI vector fragment from pGamma1 (see Figure 8). After transformation into LM1035, plasmid minipreps from resulting colonies were screened for the presence of the Bgl II - Sty1 fragment and representatives of each taken for nucleotide sequence analysis. From this, plasmids containing the desired sequence were identified and, for future reference, named as follows :

- pGamma1[L235E] containing glutamic acid at residue 235,
- pGamma1[L235A] containing alanine at residue 235,
- 10 pGamma1[G237A] containing alanine at residue 237,
- pGamma 1 [g1→g2] containing the C-gamma 2 lower hinge region.

The above plasmids were each restricted with HindIII and ApaI and the HindIII - ApaI fragment containing L243 Vh inserted to produce the following plasmids:

- L243Gamma1[L235E]
- L243Gamma1[L235A]
- L243Gamma1[G237A]
- 20 L243Gamma [g1→g2]

#### Production of chimeric L243 antibody

Antibody for biological evaluation was produced by transient expression of the appropriate heavy and light chain pairs after co-transfection into Chinese Hamster Ovary (CHO) cells using calcium phosphate precipitation.

On the day prior to transfection, semi - confluent flasks of CHO-L761 cells were trypsinised, the cells counted and T75 flasks set up each with 10exp7 cells.

On the next day, the culture medium was changed 3 hours before transfection. For transfection, the calcium phosphate precipitate was prepared by mixing 1.25 ml of 0.25M CaCl<sub>2</sub> containing 50 µg of each of heavy and light chain expression vectors with 1.25 ml of 2xHBS (16.36 gm NaCl, 11.9 gm HEPES and 0.4 gm Na<sub>2</sub>HPO<sub>4</sub> in 1 litre water with the pH

adjusted to 7.1 with NaOH) and adding immediately into the medium on the cells. After 3 hours at 37 C in a CO<sub>2</sub> incubator, the medium and precipitate were removed and the cells shocked by the addition of 15 ml 15 % glycerol in phosphate buffered saline (PBS) for 1 minute. The glycerol was removed, the cells washed once with PBS and incubated for 48 - 96 hours in 25 ml medium containing 10 mM sodium butyrate. Antibody was purified from the culture medium by binding to and elution from protein A - Sepharose. Antibody concentration was determined using a human Ig ELISA (see below).

10

### **ELISA**

For the ELISA, Nunc ELISA plates were coated overnight at 4°C with a F(ab)<sub>2</sub> fragment of a polyclonal goat anti-human Fc fragment specific antibody (Jackson Immuno-research, code 109-006-098) at 5 µg/ml in coating buffer (15mM sodium carbonate, 35mM sodium hydrogen carbonate, pH6.9). Uncoated antibody was removed by washing 5 times with distilled water. Samples and purified standards to be quantitated were diluted to approximately 1 µg/ml in conjugate buffer (0.1M Tris-HCl pH7.0, 0.1M NaCl, 0.2% v/v Tween 20, 0.2% w/v Hammersten casein). The samples were titrated in the microtitre wells in 2-fold dilutions to give a final volume of 0.1 ml in each well and the plates incubated at room temperature for 1 hr with shaking. After the first incubation step the plates were washed 10 times with distilled water and then incubated for 1 hr as before with 0.1 ml of a mouse monoclonal anti-human kappa (clone GD12) peroxidase conjugated antibody (The Binding Site, code MP135) at a dilution of 1 in 700 in conjugate buffer. The plate was washed again and substrate solution (0.1 ml) added to each well. Substrate solution contained 150 µl N,N,N,N-tetramethylbenzidine (10 mg/ml in DMSO), 150 µl hydrogen peroxide (30% solution) in 10 ml 0.1M sodium acetate/sodium citrate, pH6.0. The plate was developed for 5-10 minutes until the absorbance at 630nm was approximately 1.0 for the top standard. Absorbance at 630nm was measured using a plate reader and the concentration of the sample determined by comparing the titration curves with those of the standard.

35

**TABLE 1**

Oligonucleotide primers for the 5' region  
of mouse heavy chains.

- 5
- CH1 : 5'ATGAAATGCAGCTGGGTCAT(G,C)TTCTT3'
- 10 CH2 : 5'ATGGGATGGAGCT(A,G)TATCAT(C,G)(C,T)TCTT3'
- CH3 : 5'ATGAAG(A,T)TGTGGTTAACTGGGTTTT3'
- CH4 : 5'ATG(G,A)ACTTTGGG(T,C)TCAGCTTG(G,A)T3'
- 15 CH5 : 5'ATGGACTCCAGGCTCAATTTAGTTTT3'
- CH6 : 5'ATGGCTGTC(C,T)T(G,A)G(G,C)GCT(G,A)CTCTTCTG3'
- 20 CH7 : 5'ATGG(G,A)ATGGAGC(G,T)GG(G,A)TCTTT(A,C)TCTT3'
- CH8 : 5'ATGAGAGTGCTGATTCTTTTGTG3'
- CH9 : 5'ATGG(C,A)TTGGGTGTGGA(A,C)CTTGCTATT3'
- 25 CH10 : 5'ATGGGCAGACTTACATTCTCATTCT3'
- CH11 : 5'ATGGATTTTGGGCTGATTTTTTTTATTG3'
- 30 CH12 : 5'ATGATGGTGTTAAGTCTTCTGTACCT3'

Each of the above primers has the sequence  
5'GCGCGCAAGCTTGCCGCCACC3' added to its 5' end.

**TABLE 2**

Oligonucleotide primers for the 5' region of  
mouse light chains.

5	
	CL1 : 5'ATGAAGTTGCCTGTTAGGCTGTTGGTGCT3'
10	CL2 : 5'ATGGAG(T,A)CAGACACACTCCTG(T,C)TATGGGT3'
	CL3 : 5'ATGAGTGTGCTCACTCAGGTCCT3'
	CL4 : 5'ATGAGG(G,A)CCCCTGCTCAG(A,T)TT(C,T)TTGG3'
15	CL5 : 5'ATGGATTT(T,A)CAGGTGCAGATT(T,A)TCAGCTT3'
	CL6 : 5'ATGAGGT(T,G)C(T,C)(T,C)TG(T,C)T(G,C)AG(T,C) T(T,C)CTG(A,G)G3'
20	CL7 : 5'ATGGGC(T,A)TCAAGATGGAGTCACA3'
	CL8 : 5'ATGTGGGGA(T,C)CT(G,T)TTT(T,C)C(A,C)(A,C)TTT TTCAAT3'
25	CL9 : 5'ATGGT(G,A)TCC(T,A)CA(G,C)CTCAGTTCCTT3'
	CL10 : 5'ATGTATATATGTTTGTGTCTATTTC3'
30	CL11 : 5'ATGGAAGCCCCAGCTCAGCTTCTCTT3'

Each of the above primers has the sequence  
 5'GGACTGTTCTGAAGCCGCCACC3' added to its 5' end.

**TABLE 3****Oligonucleotide primers for the 3' ends  
of mouse Vh and Vl genes.**

5

Light chain ( CL12 ) :

5'GGATACAGTTGGTGCAGCATCCGTACGTTT3'

10 Heavy chain ( R2155 ):

5'GCAGATGGGCCCTTCGTTGAGGCTG(A,C)(A,G)GAGAC(G,T,A)GTGA3'

15

**TABLE 4****5' Primer mixtures for PCR**

20 B1 : CL2.

B2 : CL6.

B3 : CL8.

25

B4 : CL4, CL9.

B5 : CL1, CL3, CL5, CL7, CL10, CL11.

30 B6 : CH6.

B7 : CH7.

B8 : CH2, CH4.

35

B9 : CH1, CH3, CH5, CH8, CH9, CH10, CH11, CH12.



**EXAMPLE 2**

L243 is a mouse monoclonal antibody raised against human MHC Class II. The nucleotide and amino acid sequences of L243 VI and Vh are shown in Figures 1 and 2 respectively. The following examples describe the humanisation of the L243 antibody (CDR grafting).

**CDR grafting of L243 light chain**

Alignment of the framework regions of L243 light chain with those of the four human light chain subgroups [Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S. and Foeller, C. 1991, Sequences of Proteins of Immunological Interest, Fifth Edition] revealed that L243 was most homologous to antibodies in human light chain subgroup 1. Consequently, for constructing the CDR grafted light chain, the framework regions chosen corresponded to those of the human Group 1 consensus sequence. A comparison of the amino acid sequences of the framework regions of L243 and the consensus human group I light chains is given below and shows that there are 21 differences (underlined) between the two sequences.

Analysis of the contribution that any of these framework differences might have on antigen binding (see published International patent application No. WO91/09967) identified 4 residues for investigation; these are at positions 45,49,70 and 71. Based on this analysis, two versions of the CDR grafted light chain were constructed. In the first of these, L243-gL1, residues 45,49,70 and 71 are derived from the L243 light chain while in the second, L243-gL2, all residues are human consensus.

**Light Chain Residues**

Residues 37 (Gln to Arg) and 48 (Ile to Val) would be included in any future grafted molecules.

**Light chain Comparisons**

Hu group 1 consensus	: DIQMTQSPSSLSASUGDAVTITC
L243	: DIQMTQSP <u>AS</u> LS <u>SU</u> SUG <u>ET</u> UTITC

		4	4
		5	9
	Hu Group 1 consensus	:	WYQQKPGKAPKLLIY
5	L243	:	WY <u>Q</u> K <u>Q</u> GK <u>S</u> P <u>Q</u> LL <u>U</u> F
			77
			01
10	Hu Group 1 consensus	:	GUPSRFSGSGSGTDFTLTISLQPEDFATYYC
	L243	:	GUPSRFSGSGSGT <u>Q</u> YSLK <u>I</u> NSLQSEDFG <u>D</u> YYC
	Hu Group 1 consensus	:	FGQGTKVEIKR
15	L243	:	FGG <u>G</u> TNLEIKR

#### Construction of CDR grafted light chain L243-gL1

The construction of L243-gL1 is given below in detail. The following oligonucleotides were used in the Polymerase Chain Reactions (PCR) to introduce changes into the framework regions of the chimeric light chain:

R5043 : 5'GTAGGAGACCGGGTCACCATCACATGTCGAGCAA3'  
 R5044 : 5'CTGAGGAGCTTTTCTGCTGCTGATACCATGCTAAA3'  
 R5045 : 5'AAACCAGGAAAGCTCCTCAGCTCCTGATCTTTGCTGCATC3'  
 25 R5046 : 5'CTTCTGGCTGCAGGCTGGAGATAGTTAGGGTATACTGTGTGCC3'  
 R5047 : 5'CTTCAGCCTGCAGCCAGAGATTTTGCTACTTATTACTGTCAA3'  
 R5048 : 5'GGGCCGCTACCGTACGTTTTAGTTCCACTTTGGTGCCTTGACCGAA3'

Three reactions, each of 20 µl, were set up each containing 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01% w/v gelatin, 0.25 mM each deoxyribonucleoside triphosphate, 0.1 µg chimeric L243 light chain DNA, 6 pmoles of R5043/R5044 or R5045/R5046 or R5047/R5048 and 0.25 units Taq polymerase. Reactions were cycled through 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. After 30 cycles, each reaction was analysed by electrophoresis on an agarose gel and the PCR fragments excised from the gel and recovered using a Mermaid Kit (supplied by Stratech Scientific Ltd., Luton, England).

Aliquots of these were then subjected to a second round of PCR. The reaction, 100  $\mu$ l, contained 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01% w/v gelatin, 1/10 of each of the three PCR fragments from the first set of reactions, 30 pmoles of R5043 and R5048 and 2.5 units Taq polymerase. Reaction temperatures were as above. After the PCR, the mixture was extracted with phenol / chloroform and then with chloroform and precipitated with ethanol. The ethanol precipitate was recovered by centrifugation, dissolved in the appropriate buffer and restricted with the enzymes BstEII and SphI. The resulting product was finally electrophoresed on an agarose gel and the 270 base pair DNA fragment recovered from a gel slice and ligated into the vector pMR15.1 (Figure 3) that had previously been digested with the same enzymes.

The ligation mixture was used to transform E. coli LM1035 and resulting colonies analysed by PCR, restriction enzyme digests and nucleotide sequencing. The nucleotide and amino acid sequence of the VI region of L243-gL1 is shown in Figure 4.

#### Construction of CDR grafted light chain L243-gL2

L243-gL2 was constructed from L243-gL1 using PCR. The following oligonucleotides were used to introduce the amino acid changes :

R1053 : 5'GCTGACAGACTAACAGACTGTTCC3'

R5350 :

5'TCTAGATGGCACCCATCTGCTAAGTTTGATGCAGCATAGATCAGGAGCTTAGGAGC3'

R5349 :

5'GCAGATGGTGTGCCATCTAGATTCAGTGGCAGTGGATCAGGCACAGACTTTACCTAAC3'

R684 : 5'TTCAACTGCTCATCAGAT3'

Two reactions, each 20  $\mu$ l, were set up each containing 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01% w/v gelatin, 0.25 mM each deoxyribonucleoside tri-phosphate, 0.1  $\mu$ g L243-gL1, 6 pmol of R1053/R5350 or R5349/R684 and 0.25 units Taq polymerase. Reactions

were cycled through 94°C for 1 minute, 55 C for 1 minut and 72°C for 1 minute. After 30 cycles, each reaction was analysed by electrophoresis on an agarose gel and the PCR fragments excised from the gel and recovered using a Mermaid Kit.

5

Aliquots of these were then subjected to a second round of PCR. The reaction, 100 µl, contained 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01% w/v gelatin, 1/5 of each of the PCR fragments from the first set of reactions, 30 pmoles of R1053 and R684 and 2.5 units Taq polymerase.

10 Reaction temperatures were as above. After the PCR, the mixture was extracted with phenol/ chloroform and then with chloroform and precipitated with ethanol. The ethanol precipitate was recovered by centrifugation, dissolved in the appropriate buffer and restricted with the enzymes BstEII and SphI. The resulting product was finally  
15 electrophoresed on an agarose gel and the 270 base pair DNA fragment recovered from a gel slice and ligated into the vector pMR15.1 (Figure 3) that had previously been digested with the same enzymes.

The ligation mixture was used to transform E. coli LM1035 and resulting  
20 colonies analysed by PCR, restriction enzyme digests and nucleotide sequencing. The nucleotide and amino acid sequence of the VI region of L243-gL2 is shown in Figure 5.

#### **CDR grafting of L243 heavy chain**

25 CDR grafting of L243 heavy chain was accomplished using the same strategy as described for the light chain. L243 heavy chain was found to be most homologous to human heavy chains belonging to subgroup 1 and therefore the consensus sequence of the human subgroup 1 frameworks was chosen to accept the L243 heavy chain CDRs.

30

A comparison of the framework regions of the two structures is shown below where it can be seen that L243 differs from the human consensus at 28 positions (underlined). After analysis of the contribution that any of these might make to antigen binding, only residues 27, 67, 69, 71, 72, and  
35 75 were retained in the CDR grafted heavy chain, L243-gH.

## Heavy Chain Residues

Residues 2 (Val to Ile) and 46 (Glu to Lys) would be incorporated into any future grafted molecules. In addition to these two residues the murine residue 67 which is present in L243 gH would be changed back to the human consensus residue i.e. Phe to Val change.

### Heavy chain comparisons

2  
7

10 Hu Group 1 consensus : QVQLVQSGAEVKKPGASVKVSCKASGYTFT  
L243 : QIQLVQSGPELKKPGETVKISCKASGFTFT

Hu Group 1 consensus : WVAQAPGQGLEWMG  
L243 : WVQAPGKGLKWMG

15 6 6 7 7 7  
7 9 12 5

Hu Group 1 consensus : RVTITADTSTSTAYMELSSLASEDTAVYYCAR  
L243 : RFAFSLETSASTAYLQINNLKNEDTAKYFCAR

20 Hu Group 1 consensus : WGQGTLLTVSS  
L243 : WGQGTLLTVSS

### Construction of CDR grafted heavy chain, L243 qH

25 L243gH was assembled by subjecting overlapping oligonucleotides to PCR in the presence of the appropriate primers. The following oligonucleotides were used in the PCR:

R3004 : 5'GGGGGGGAGCTTGCCGCCACCATGG3'

30 R3005 : 5'CCCCCGGGCCCTTTGTAGAGCAG3'

R4902 : 5'GACACAGGAGTGCACTCTCAGGTGCAGCTGGTGCAGTCTGGAGC  
AGAGGTGAGAGAGCCTGGAGCATCTG3'

35 R4903 : 5'ACATTCACAAATTACGGAATGAATTGGGTGAGACAGGCACCTGGA  
CAGGGACTCGAGTGGG3'

R4904 : 5'CCTACGTACGCAGACGACTTCAAGGGAGGATTCACATTCACACTG  
GAGACATCTGCATCTACAGCATACAT3'

R4905 : 5'CAGCAGTGTA CTACTGTGCAAGAGACATTACAGCAGTGGTACCTA  
CAGGATTCGACTACTGGGGACAGGGA3'

5 R4897 : 5'TGAGAGTGCACTCCTGTTGTCACAGACAGGAAGAACAGGAACACC  
CAAGACCACTCCATGGTGGCGGCAAGCTTCCCCC3'

R4898 : 5'CATTCCGTAA TTTGTGAATGTGAATCCAGATGCCTTACAGACAC  
CTTCACAGATGCTCCAGGCTTCTTCA3'

10 R4899 : 5'GAAGTCGTCTGCGTACGTAGGCTCTCTTGTGTATGTATTATCCA  
TCCCATCCACTCGAGTCCCTGTCCAG3'

15 R4900 : 5'TTGCA CAGTAGTACACTGCTGTGTCCTCAGATCTCAGAGAAGACA  
GCTCCATGTATGCTGTAGATGCAGAT3'

R4901 : 5'CCCCCGGGCCCTTTGTAGAGCAGAGACACTGTCACCAGTGTT  
CCCTGTCCCCAGTAGTCGA3'

20 The assembly reaction, 50  $\mu$ l, contained 10 mM Tris-HCl pH 8.3, 1.5 mM  
MgCl<sub>2</sub>, 50 mM KCl, 0.01% w/v gelatin, 0.25 mM each deoxyribo-  
nucleoside triphosphate, 1 pmole of each of R4897 - R4905, 10 pmoles of  
each of R3004 and R3005 and 2.5 units Taq polymerase. Reactions were  
cycled through 94°C for 1 minute, 55°C for 1 minute and 72°C for 1  
25 minute. After 30 cycles, the reaction was extracted with phenol/chloroform  
(1/1), then with chloroform and precipitated with ethanol. After  
centrifugation, the DNA was dissolved in the appropriate restriction buffer  
and digested with HindIII and Apal. The resulting fragment was isolated  
30 from an agarose gel and ligated into pMR14 (Figure 6) that had previously  
been digested with the same enzymes. pMR14 contains the human  
gamma 4 heavy chain constant region and so the heavy chain expressed  
from this vector will be a gamma 4 isotype. The ligation mixture was used  
to transform E. coli LM1035 and resulting bacterial colonies screened by  
restriction digest and nucleotide sequence analysis. In this way, a plasmid  
35 containing the correct sequence for L243gH was identified (Figure 7).

Construction of Gamma 1 versions of chimeric and CDR grafted L243  
heavy chain

Human Gamma 1 versions of L243 heavy chains were constructed by transferring the variable regions of both the murine and the CDR grafted heavy chains as HindIII to ApaI fragments into the vector pGamma1 (Figure 8). This vector contains the human Gamma 1 heavy chain constant region.

#### Evaluation of activities of CDR grafted genes

The activities of the CDR grafted genes were evaluated by expressing them in mammalian cells and purifying and quantitating the newly synthesised antibodies. The methodology for this is described next, followed by a description of the biochemical and cell based assays used for the biological characterisation of the antibodies.

##### a) Gene Expression in CHO cells

Chimeric and CDR grafted L243 was produced for biological evaluation by transient expression of heavy and light chain pairs after co-transfection into Chinese Hamster Ovary (CHO) cells using calcium phosphate precipitation.

On the day prior to transfection, semi-confluent flasks of CHO-L761 cells [Cockett, M. I., Bebbington, C. R. and Yarranton, G. T. 1991, Nucleic Acids Research 19, 319-325] were trypsinised, the cells counted and T75 flasks set up each with  $10^7$  cells. On the next day, the culture medium was changed 3 hours before transfection. For transfection, the calcium phosphate precipitate was prepared by mixing 1.25 ml of 0.25M  $\text{CaCl}_2$  containing 50  $\mu\text{g}$  of each of heavy and light chain expression vectors with 1.25 ml of 2xHBS ( 16.36 gm NaCl, 11.9 gm HEPES and 0.4 gm  $\text{Na}_2\text{HPO}_4$  in 1 litre water with the pH adjusted to 7.1 with NaOH ) and adding immediately into the medium on the cells. After 3 hours at  $37^\circ\text{C}$  in a  $\text{CO}_2$  incubator, the medium and precipitate were removed and the cells shocked by the addition of 15 ml 15 % glycerol in phosphate buffered saline (PBS ) for 1 minute. The glycerol was removed, the cells washed once with PBS and incubated for 48 - 96 hours in 25 ml medium containing 10 mM sodium butyrate. Antibody was purified from the culture medium by binding to and elution from proteinA - Sepharose. Antibody concentration was determined using a human Ig ELISA (see below).

**b) ELISA**

For the ELISA, Nunc ELISA plates were coated overnight at 4°C with a F(ab)<sub>2</sub> fragment of a polyclonal goat anti-human Fc fragment specific antibody (Jackson Immuno-research, code 109-006-098) at 5 µg/ml in coating buffer (15mM sodium carbonate, 35mM sodium hydrogen carbonate, pH6.9). Uncoated antibody was removed by washing 5 times with distilled water. Samples and purified standards to be quantitated were diluted to approximately 1 µg/ml in conjugate buffer (0.1M Tris-HCl pH7.0, 0.1M NaCl, 0.2% v/v Tween 20, 0.2% w/v Hammersten casein). The samples were titrated in the microtitre wells in 2-fold dilutions to give a final volume of 0.1 ml in each well and the plates incubated at room temperature for 1 hr with shaking. After the first incubation step the plates were washed 10 times with distilled water and then incubated for 1 hr as before with 0.1 ml of a mouse monoclonal anti-human kappa (clone GD12) peroxidase conjugated antibody (The Binding Site, code MP135) at a dilution of 1 in 700 in conjugate buffer. The plate was washed again and substrate solution (0.1 ml) added to each well. Substrate solution contained 150 µl N,N,N,N-tetramethylbenzidine (10 mg/ml in DMSO), 150 µl hydrogen peroxide (30% solution) in 10 ml 0.1M sodium acetate/sodium citrate, pH6.0. The plate was developed for 5-10 minutes until the absorbance at 630nm was approximately 1.0 for the top standard. Absorbance at 630nm was measured using a plate reader and the concentration of the sample determined by comparing the titration curves with those of the standard.

**c) Competition Assay**

The principle of this assay is that if the antigen binding region has been correctly transferred from the murine to human frameworks, then the CDR grafted antibody will compete equally well with a labelled chimeric antibody for binding to human MHC Class II. Any changes in the antigen binding potency will be revealed in this system.

Chimeric L243 was labelled with fluorescein (FITC) using the method of Wood et al [Wood, T., Thompson, S and Goldst in, G (1965), J. Immunol 95, 225-229]. All dilutions, manipulations and incubations were done in



phosphate buff red saline (PBS, Gibco, UK) containing 0.1% sodium azide and 5% Fetal Calf serum (Sigma, UK). Serial dilutions of antibodies in 100µl in RB polystyrene tubes (2052 12x75mm Falcon, UK) were premixed with a constant amount of the FITC-labelled antibody (at a  
5 previously determined optimal concentration) and added to  $5 \times 10^4$  indicator cells (JY B lymphoblastoid cell line bearing high levels of HLA-DR). Cells and antibody were incubated together at 4°C for 30 minutes, washed twice and binding revealed using a Fluorescence Activated Cell Scanner (FACS Becton Dickinson). After appropriate analysis, median  
10 fluorescence intensity is plotted against antibody concentration.

Figure 9 compares the ability of combinations of L243 heavy and light chains to compete with FITC-labelled chimeric L243 for binding to JY cells. All combinations were effective competitors although none of those  
15 containing CDR grafted heavy or light chains were as effective as the chimeric antibody itself. Thus, the combinations cH/gL1, gH/cL and gH/gL1 were 89%, 78% and 64% respectively, as effective as chimeric L243 in this assay.

20 d) Determination of Affinity constants by Scatchard Analysis

L243 antibodies were titrated from 10µg/ml in PBS, 5% fetal calf serum, 0.1% sodium azide in 1.5-fold dilutions (150µl each) before incubation with  $5 \times 10^4$  JY cells per titration point for 1 hour on ice. The cells were previously counted, washed and resuspended in the same medium as the  
25 samples. After incubation, the cells were washed with 5ml of the above medium, spun down and the supernatant discarded. Bound antibody was revealed by addition of 100µl of a 1/100 dilution of FITC conjugated anti-human Fc monoclonal (The Binding Site; code MF001). The cells were then incubated for 1 hour on ice and then the excess FITC conjugate  
30 removed by washing as before. Cells were dispersed in 250µl of the same buffer and the median fluorescence intensity per cell was determined in a FACScan (Becton Dickinson) and calibrated using standard beads (Flow Cytometry standards Corporation). The number of molecules of antibody bound per cell at each antibody concentration was thus established and  
35 used to generate Scatchard plots. For the purpose of calculation, it was

assumed that the valency of binding of the FITC conjugate to L243 was 1:1 and that the F/P ratio was 3.36 (as given by the manufacturer).

5 A Scatchard plot comparing the affinities of chimeric L243 (cH/cL), L243-gH/L243-gL1 and L243-gH/L243-gL2 is shown in Figure 10. Chimeric L243 was found to have an apparent  $K_d$  of 4.1 nM while the CDR grafted antibodies containing gL1 and gL2 light chains had apparent  $K_d$  of 6.4nM and 9.6nM respectively. The difference in  $K_d$  values of the antibodies with the two CDR grafted light chains reflects the contribution made by  
10 residues 45,49,70 and 71 that had been retained, in L243-gL1, from the parent light chain.

e) Antibody dependent cell mediated cytotoxicity.

15 The ability of chimeric and CDR grafted L243 to mediate antibody dependent cell cytotoxicity (ADCC) was compared. The principle of the experiment is that antibodies will mediate lysis of target cells bearing their cognate antigen if the Fc of the antibody is able to interact with Fc receptor bearing effector cells capable of cytotoxicity.

20 Effector cells are prepared fresh for each experiment. Human venous blood is drawn into endotoxin free tubes containing heparin. Peripheral blood mononuclear cells (PBMC) are prepared by density gradient centrifugation according to the manufacturers instructions (Pharmacia). PBMCs are adjusted to  $1 \times 10^7$  cells/ml in RPMI 1640 medium (Gibco)  
25 containing 2 mM glutamine (Gibco, UK) and 10% fetal calf serum, in which all manipulations, dilutions are incubations are done.

Target cells (JY, see above) are labelled with 1mCi  $\text{Na}^{51}\text{Cr}$  for 1 hour at room temperature, agitated every 15 minutes. The cells are then washed  
30 three times to remove free radiolabel and resuspended at  $2 \times 10^6$  cells/ml. Serial antibody dilutions are prepared in duplicate in sterile U-bottomed 96 well microtitre plates (Falcon, UK) in 25 $\mu\text{l}$ . Control wells containing medium only are also prepared to establish the spontaneous release of label giving the assay background. Target  $^{51}\text{Cr}$  labelled JY cells are  
35 added to all wells in 10 $\mu\text{l}$ . The same number of JY cells are also added to wells containing 2% Triton X100 in water to establish the 100% release

value. Target cells and antibody are incubated together and, after 30 minutes at room temperature, 25µl effector cells are added to all wells (except the 100%) for a further 4 hours at 37°C. 100µl of EDTA/saline at 4°C is then added to stop any further cell killing, the microtitre plates are  
5 centrifuged at 200xg to pellet the intact cells and 100µl of the supernatant is removed and counted in a gamma counter.

Percent cell lysis is calculated by subtracting the background from all values and then expressing them as a percentage of the adjusted  
10 maximum release. Replicates vary by less than 5%. Percent cell lysis is then plotted against antibody concentration.

A comparison of the activities of chimeric (cH/cL) and CDR grafted (gH/gL1) L243 human gamma 1 isotypes in the above assay is shown in  
15 Figure 11. Both antibodies were effective mediators of cell lysis with maximal activity being achieved at antibody concentrations of less than 100 ng/ml. There was no significant difference between the activities of the two antibodies.

#### 20 f) Immune function tests

Ex vivo T cell function experiments were performed where an interaction between MHC-II and the T cell receptor was an obligatory requirement for T cell activation. Chimeric and CDR grafted L243 antibodies were compared in mixed lymphocyte reactions, which measures both naive and  
25 memory T cell activation, and in recall responses to tetanus toxoid which only measures a memory T cell response.

##### 1) Mixed Lymphocyte reaction

The principle of the experiment is that when leucocytes from one individual  
30 are mixed with those of another individual which express different HLA alleles, they will recognise each other as foreign and the lymphocytes will become activated. This activation is dependent primarily on interactions between the CD3/TcR complex on T cells and the MHC Class II molecule on antigen presenting cells. L243 is known to inhibit this reaction.

Leucocytes are prepared fresh for each experiment. Human venous blood from two individuals is drawn into endotoxin free tubes containing heparin. Peripheral blood mononuclear cells (PBMC) are prepared by density centrifugation according to the manufacturers instructions (Pharmacia).  
5 PBMC are adjusted to  $2 \times 10^6$  cells/ml in RPMI 1640 medium (Gibco, UK) containing 2 mM glutamine, 100 µg/ml penicillin/streptomycin and 10% fetal calf serum, in which all manipulations, dilutions and incubations are done. PBMC from one individual are irradiated with 3000 rads. These cells will be used to stimulate a response from those of the other individual.

10

Serial dilutions of antibodies are prepared in triplicate in sterile U-bottom 96 well microtitre plates (Falcon, UK) in 100µl. Control wells containing either medium alone or 100nM cyclosporin (Sandimmun, Sandoz) are also prepared to establish the maximum response and maximum inhibition  
15 respectively. Equal numbers of irradiated stimulators and responders are mixed together and 100µl is added to each well. Wells with stimulator alone and responders alone are also set up as controls. The experiment is incubated at 37°C in 100% humidity and 5% CO<sub>2</sub> for 5 days. Response is measured by assessing cell proliferation during the last 18 hours of culture  
20 by incubation with 1 µCi/well <sup>3</sup>H-Thymidine (Amersham, UK), harvesting onto glass filter mats and counting using a beta counter.

When an MLR was carried out to compare the effectiveness of the Gamma 1 isotypes of chimeric and CDR grafted L243 as inhibitors of T  
25 cell activation, no significant differences were observed between the two antibodies (Figure 12). Greater than 90% inhibition of the MLR was observed using 100 ng/ml of either antibody.

## 2) T cell recall response to Tetanus toxoid

30 The ability of chimeric and CDR grafted L243 to suppress a secondary response was assessed using a recall response to Tetanus toxin. The principle of the experiment is that T lymphocytes from an individual previously immunised with Tetanus toxoid (TT) will respond to TT when re-exposed ex vivo. This activation is dependent on the interaction between  
35 the CD3/TcR complex on T cells and the MHC Class II molecules on cells

which process and present the antigen. L243 is known to inhibit this reaction.

PBMC are prepared as described above. Serial dilutions of antibodies are prepared in triplicate in sterile U-bottom 96 well microtitre plates in 100µl. 50µl containing an optimal concentration of TT, previously determined by experimentation, is added to all wells. Control wells containing medium only or 100nM cyclosporin are also prepared to establish the maximum response and maximum inhibition, respectively. 50µl PBMC are then added to each well. The experiment is incubated at 37°C in 100% humidity and 5% CO<sub>2</sub> for 7 days. Response is measured by assessing cell proliferation during the last 18 hours of culture by incubation with 1µCi/well <sup>3</sup>H-thymidine, harvesting onto glass filter mats and counting using a beta counter.

15

The results of an experiment comparing the ability of human gamma 1 isotypes of chimeric and CDR grafted L243 to inhibit the response to TT is shown in Figure 13. Both antibodies were effective inhibitors of the T cell response to TT and produced titration curves that were indistinguishable.

20

### **EXAMPLE 3**

The ability of CDR grafted L243 with the alteration at position 235 i.e. [L235E] to mediate antibody dependent cell cytotoxicity (ADCC) was measured essentially as described in Example 2. The results are shown in Figure 15.

25

Similarly the CDR grafted L243 [L235E] antibody was tested in a mixed lymphocyte reaction and in recall response to tetanus toxoid essentially as described in Example 2. The results are provided in Figures 16 and 17.

30

### **ANTIBODY DEPENDENT COMPLEMENT MEDIATED CYTOTOXICITY**

The ability of the engineered variants of L243 to fix human complement was assessed using the technique of antibody dependent complement mediated cytotoxicity.

35

The principle of the experiment is that antibodies will mediate complement lysis of target cells bearing their cognate antigen if the Fc of the antibody is able to interact with the components of the (usually classical) complement cascade. The critical interaction is with the C1q molecule.

5

The source of complement in these experiments is human venous blood freshly drawn into endotoxin free glass bottles which is then allowed to clot at 37°C for 1 hour. The clot is detached from the glass and then incubated at 4°C for 2 hours to allow it to retract. The clot is then removed and the serum separated from the remaining red cells by centrifugation at 1000g. Once prepared, the serum can be stored for up to one month at -20°C without noticeable deterioration of potency.

10

All manipulations, dilutions and incubations are done in RPMI 1640 medium (Gibco UK) containing 2mM Glutamine (Gibco UK) and 10% foetal calf serum (Sigma UK). Target cells (JY B lymphoblastoid cell line bearing high levels of HLA-DR) are labelled with 1mCi Na<sup>51</sup>Cr for 1 hour at room temperature, agitated every 15 min. The cells are then washed three times, to remove free radiolabel, and resuspended at 2x10<sup>6</sup>/ml. Serial antibody dilutions are prepared in duplicate in V-bottom 96 well microtitre plates (ICN/Flow UK) in 25ml. Control wells containing medium only are also prepared to establish the spontaneous release of label giving the assay back-ground. Target <sup>51</sup>Cr labelled JY cells are added to all wells in 10ml. The same number of JY cells are also added to wells containing 2% Triton x100 in water to establish the 100% release value. Target cells and antibody are incubated together and, after 1 hour at room temperature, 25ml serum as a source of complement is added to all wells (except the 100%) for a further 1 hour at room temperature. 100ml of EDTA saline at 4°C is then added to stop any further cell killing, the microtitre plates are centrifuged at 200g to pellet the intact cells and 100ml supernatant is removed and counted in a gamma counter.

20

25

30

Percent cell lysis is calculated by subtracting the background from all values and then expressing them as a percentage of the adjusted maximum release. Replicates vary by less than 5%. Percent cell lysis is then plotted against antibody dilution,

35

The results (without subtraction of background) are shown in Figure 18.

**CLAIMS**

- 5 1. A recombinant antibody molecule having specificity for antigenic determinants dependent on the DR $\alpha$  chain.
2. A recombinant antibody molecule according to Claim 1 having specificity for the epitope recognised by the murine monoclonal antibody L243.
- 10 3. A recombinant antibody molecule according to Claim 1 or Claim 2 which is a humanised CDR-grafted or chimeric antibody molecule.
- 15 4. A humanised antibody molecule according to Claim 3 wherein at least one of the complementarity determining regions (CDRs) of the variable domain is derived from the mouse monoclonal antibody L243 and the remaining immunoglobulin-derived parts of the humanised antibody molecule are derived from a human immunoglobulin or an analogue thereof.
- 20 5. A CDR-grafted humanised antibody heavy chain according to Claim 4 having a variable region domain comprising human acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one or more of
- 25 positions 27, 67, 69, 71, 72 and 75.
6. A CDR-grafted humanised antibody heavy chain according to Claim 5 comprising donor residues at positions 27, 67, 69, 71, 72 and 75.
- 30 7. A CDR-grafted humanised antibody heavy chain having a variable region domain comprising human acceptor frameworks derived from human group consensus sequence 1 and L243 donor antigen binding regions wherein the framework comprises L243 donor residues at one of more of positions 27, 67, 69, 71, 72 and 75.



- 5 8. A CDR-grafted humanised antibody light chain according to Claim 4 having a variable region domain comprising human acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one or more of positions 45, 49, 70 and 71.
9. A CDR-grafted humanised antibody light chain according to Claim 7 comprising donor residues at positions 45, 49, 70 and 71.
- 10 10. A CDR-grafted humanised antibody light chain having a variable region domain comprising acceptor frameworks derived from human group consensus sequence 1 and L243 donor antigen binding regions wherein the framework comprises L243 donor residues at one or more of positions 45, 49, 70 and 71.
- 15 11. A DNA sequence which codes for a CDR-grafted heavy chain according to any one of Claims 5 or 6 and/or a CDR-grafted light chain according to any one of Claims 8 or 9.
- 20 12. A cloning or expression vector containing a DNA sequence according to Claim 11.
13. A host cell transformed with a cloning or expression vector according to Claim 12.
- 25 14. A process for the production of a CDR-grafted antibody having specificity for the epitope recognised by the murine monoclonal antibody L243 comprising expressing a DNA sequence according to Claim 11 or 12 in a transformed host cell.
- 30 15. A process for producing a recombinant or humanised antibody having specificity for the epitope recognised by the murine monoclonal antibody L243 comprising:
- 35 a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy or light chain comprising a variable domain wherein at least one of the CDRs of

the variable domain is derived from the L243 MAb and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin

- 5           b)     producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light or heavy chain comprising a variable domain wherein at least one of the CDRs of the variable domain is derived from the MAb L243 and the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin
- 10          c)     transfecting a host cell with both operons; and
- d)     culturing the transfected cell line to produce the humanised antibody molecule.
- 15          16.    A therapeutic, pharmaceutical or diagnostic composition comprising a recombinant antibody molecule according to Claim 1 or Claim 2 in combination with a pharmaceutically acceptable carrier, diluent or excipient.
- 20          17.    A process for the preparation of a therapeutic, pharmaceutical or diagnostic composition comprising admixing a recombinant antibody molecule according to Claim 1 or Claim 2 with a pharmaceutically acceptable excipient diluent or carrier.
- 25          18.    A method of therapy or diagnosis comprising administering an effective amount of a recombinant antibody molecule according to Claim 1 or Claim 2 to a human or animal subject.

1 / 20

## FIG. 1

TTCGAGCCGCCACC ATG AGG TGC TCT GCT GAG TTT CTG GGG TTG CTG  
                   M   R   C   S   A   E   F   L   G   L   L>

CTG CTG TGG CTT ACA GAT GCC AGA TGT GAC ATC CAG ATG ACT CAG  
  L   L   W   L   T   D   A   R   C   D   I   Q   M   T   Q>

TCT CCA GCC TCC CTA TCT GTA TCT GTG GGA GAA ACT GTC ACC ATC  
  S   P   A   S   L   S   V   S   V   G   E   T   V   T   I>

ACA TGT CGA GCA AGT GAG AAT ATT TAC AGT AAT TTA GCA TGG TAT  
  T   C   R   A   S   E   N   I   Y   S   N   L   A   W   Y>

CGT CAG AAA CAG GGA AAA TCT CCT CAG CTC CTG GTC TTT GCT GCA  
  R   Q   K   Q   G   K   S   P   Q   L   L   V   F   A   A>

TCA AAC TTA GCA GAT GGT GTG CCA TCA AGG TTC AGT GGC AGT GGA  
  S   N   L   A   D   G   V   P   S   R   F   S   G   S   G>

TCA GGC ACA CAG TAT TCC CTC AAG ATC AAC AGC CTG CAG TCT GAA  
  S   G   T   Q   Y   S   L   K   I   N   S   L   Q   S   E>

GAT TTT GGG GAT TAT TAC TGT CAA CAT TTT TGG ACT ACT CCG TGG  
  D   F   G   D   Y   Y   C   Q   H   F   W   T   T   P   W>

GCG TTC GGT GGA GGC ACC AAC CTG GAA ATC AAA CGT  
  A   F   G   G   G   T   N   L   E   I   K   A>

2 / 20

## FIG. 2

AAGCTTGCCGCCACC ATG GCT TGG GTG TGG AAC TTG CTA TTC CTG ATG  
M A W U W N L L F L N>

GCA GCT GCC CAA AGT GCC CAA GCA CAG ATC CAG TTG GTG CAG TCT  
A A A Q S A Q A Q I Q L U Q S>

GGA CCT GAG CTG AAG AAG CCT GGA GAG ACA GTC AAG ATC TCC TGC  
G P E L K K P G E T U K I S C>

AAG GCT TCT GGG TTT ACC TTC ACA AAC TAT GGA ATG AAC TGG GTG  
K A S G F T F T N Y G M N W U>

AAG CAG GCT CCA GGA AAG GGT TTA AAG TGG ATG GGC TGG ATA AAC  
K Q A P G K G L K W M G W I N>

ACC TAC ACT AGA GAG CCA ACA TAT GCT GAT GAC TTC AAG GGA CGG  
T Y T A E P T Y A D D F K G R>

TTT GCC TTC TCT TTG GAA ACC TCT GCC AGC ACT GCC TAT TTG CAG  
F A F S L E T S A S T A Y L Q>

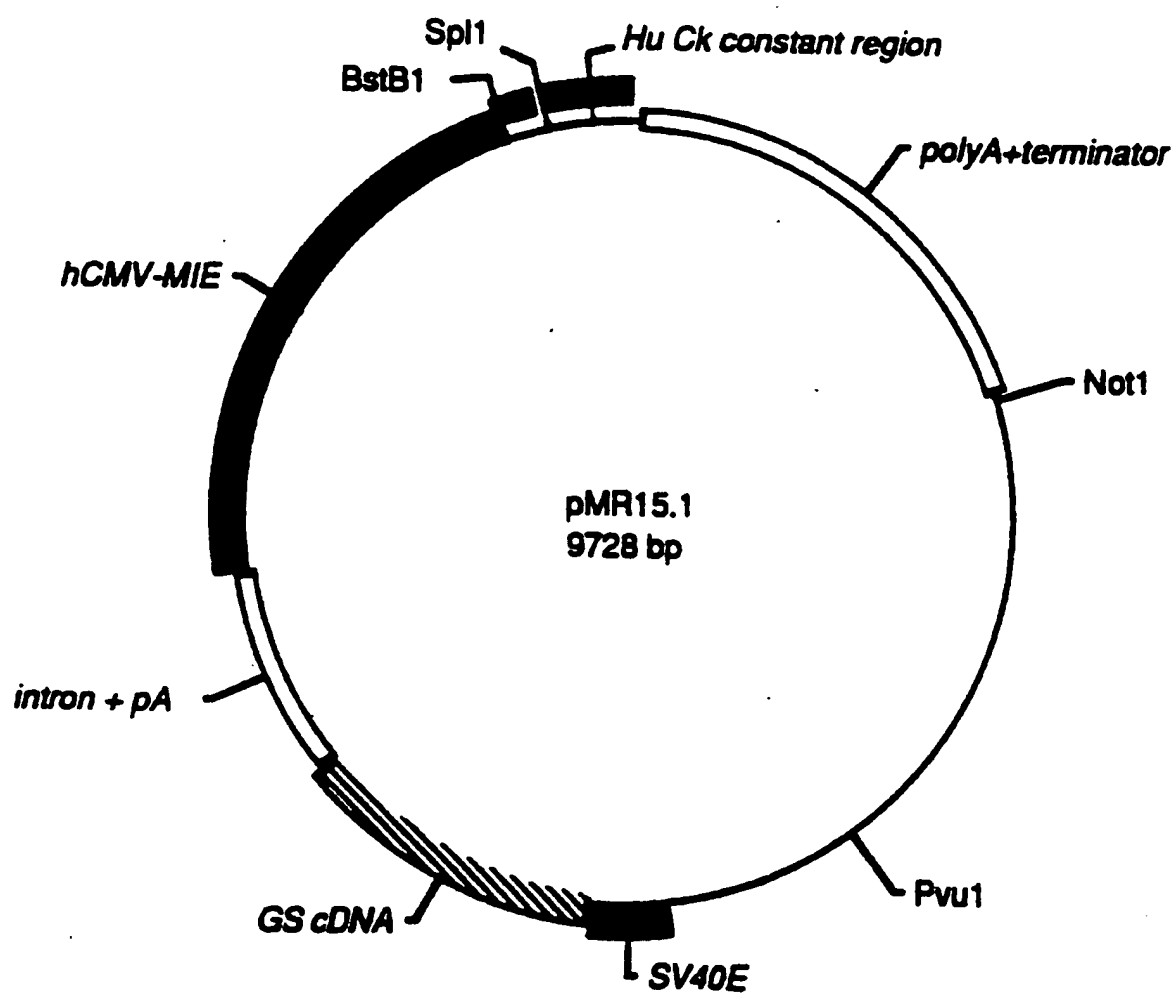
ATC AAC AAC CTC AAA AAT GAG GAC ACG GCT AAA TAT TTC TGT GCA  
I N N L K N E D T A K Y F C A>

AGA GAT ATT ACT GCG GTT GTA CCT ACG GGT TTT GAC TAC TGG GGC  
R D I T A U U P T G F D Y W G

CAA GGC ACC ACT CTC ACC GTC TCC TCA  
Q G T T L T U S S>

3 / 20

FIG. 3



4 / 20

## FIG. 4

TTCGAAGCCGCCACC ATG TCT GTC CCC ACC CAA GTC CTC GGT CTC CTG  
M S U P T Q U L G L L>

CTG CTG TGG CTT ACA GAT GCC AGA TGT GAC ATT CAA ATG ACC CAG  
L L W L T D A R C D I Q M T Q>

AGC CCA TCC AGC CTG AGC GCA TCT GTA GGA GAC CGG GTC ACC ATC  
S P S S L S A S U G D R U T I>

ACA TGT CGA GCA AGT GAG AAT ATT TAC AGT AAT TTA GCA TGG TAT  
T C R A S E N I Y S N L A W Y>

CAG CAG AAA CCA GGA AAA GCT CCT CAG CTC CTG ATC TTT GCT GCA  
Q Q K P G K A P Q L L I F A A>

TCA AAC TTA GCA GAT GGT GTG CCA TCA AGG TTC AGT GGC AGT GGA  
S N L A D G U P S R F S G S G>

TCA GGC ACA CAG TAT ACC CTA ACT ATC TCC AGC CTG CAG CCA GAA  
S G T Q Y T L T I S S L Q P E>

GAT TTT GCT ACT TAT TAC TGT CAA CAT TTT TGG ACT ACT CCG TGG  
D F A T Y Y C Q H F W T T P W>

GCG TTC GGT CAA GGC ACC AAA GTG GAA ATC AAA CGT  
A F G Q G T K U E I K R>

5 / 20

## FIG. 5

TTCGAAGCCGCCACC ATG TCT GTC CCC ACC CAA GTC CTC GGT CTC CTG  
M S U P T Q U L G L L>

CTG CTG TGG CTT ACA GAT GCC AGA TGT GAC ATT CAA ATG ACC CAG  
L L W L T D A R C D I Q M T Q>

AGC CCA TCC AGC CTG AGC GCA TCT GTA GGA GAC CGG GTC ACC ATC  
S P S S L S A S U G D R U T I>

ACA TGT CGA GCA AGT GAG AAT ATT TAC AGT AAT TTA GCA TGG TAT  
T C R A S E N I Y S N L A W Y>

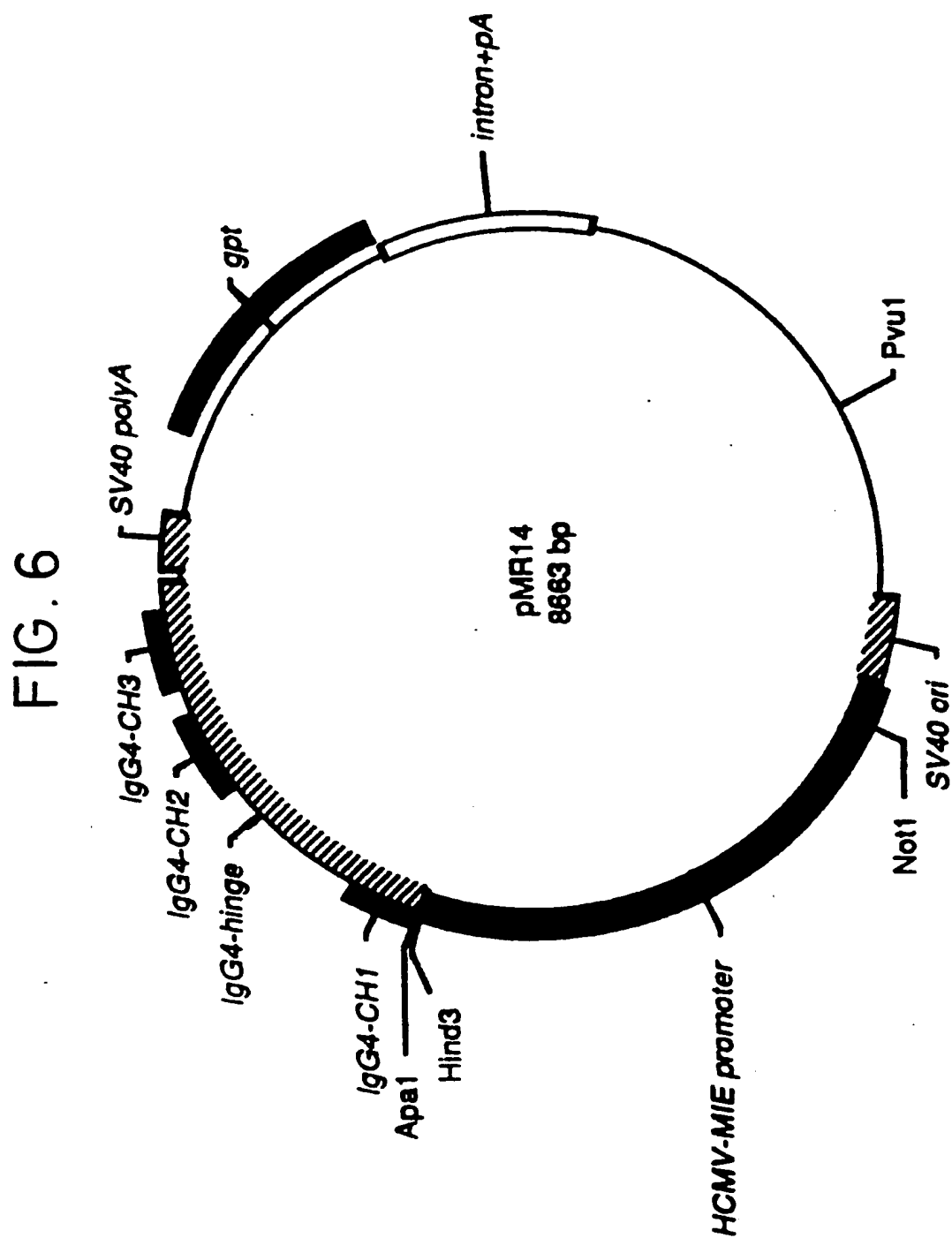
CAG CAG AAA CCA GGA AAA GCT CCT AAG CTC CTG ATC TAT GCT GCA  
Q Q K P G K A P K L L I Y A A>

TCA AAC TTA GCA GAT GGT GTG CCA TCT AGA TTC AGT GGC AGT GGA  
S N L A D G U P S R F S G S G>

TCA GGC ACA GAC TTT ACC CTA ACT ATC TCC AGC CTG CAG CCA GAA  
S G T D F T L T I S S L Q P E>

GAT TTT GCT ACT TAT TAC TGT CAA CAT TTT TGG ACT ACT CCG TGG  
D F A T Y Y C Q H F W T T P W>

GCG TTC GGT CAA GGC ACC AAA GTG GAA ATC AAA CGT  
A F G Q G T K U E I K R>





7 / 20

## FIG. 7

AAGCTTGCCGCCACC ATG GAG TGG TCT TGG GTG TTC CTG TTC TTC CTG  
 M E W S W U F L F F L>

TCT GTG ACA ACA GGA GTG CAC TCT CAG GTG CAG CTG GTG CAG TCT  
 S U T T G U H S Q U Q L U Q S>

GGA GCA GAG GTG AAG AAG CCT GGA GCA TCT GTG AAG GTG TCT TGT  
 G A E U K K P G A S U K U S C>

AAG GCA TCT GGA TTC ACA TTC ACA AAT TAC GGA ATG AAT TGG GTG  
 K A S G F T F T N Y G M N W U>

AGA CAG GCA CCT GGA CAG GGA CTC GAG TGG ATG GGA TGG ATT AAT  
 R Q A P G Q G L E W M G W I N>

ACA TAC ACA AGA GAG CCT ACG TAC GCA GAC GAC TTC AAG GGA AGA  
 T Y T A E P T Y A D D F K G R>

TTC ACA TTC ACA CTG GAG ACA TCT GCA TCT ACA GCA TAC ATG GAG  
 F T F T L E T S A S T A Y M E>

CTG TCT TCT CTG AGA TCT GAG GAC ACA GCA GTG TAC TAC TGT GCA  
 L S S L R S E D T A U Y Y C A>

AGA GAC ATT ACA GCA GTG GTA CCT ACA GGA TTC GAC TAC TGG GGA  
 R D I T A U U P T G F D Y W G>

CAG GGA ACA CTG GTG ACA GTG TCT TCT  
 Q G T L U T U S S>

FIG. 8

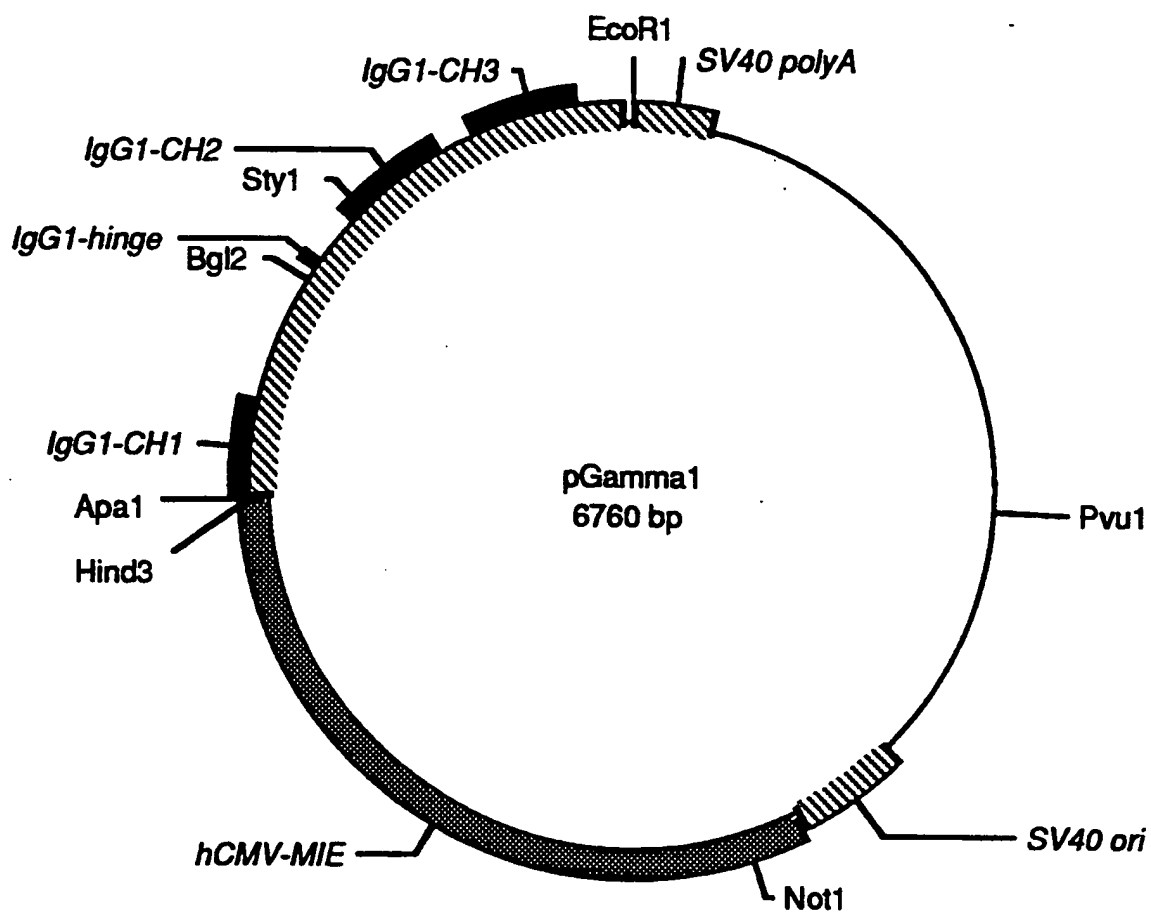
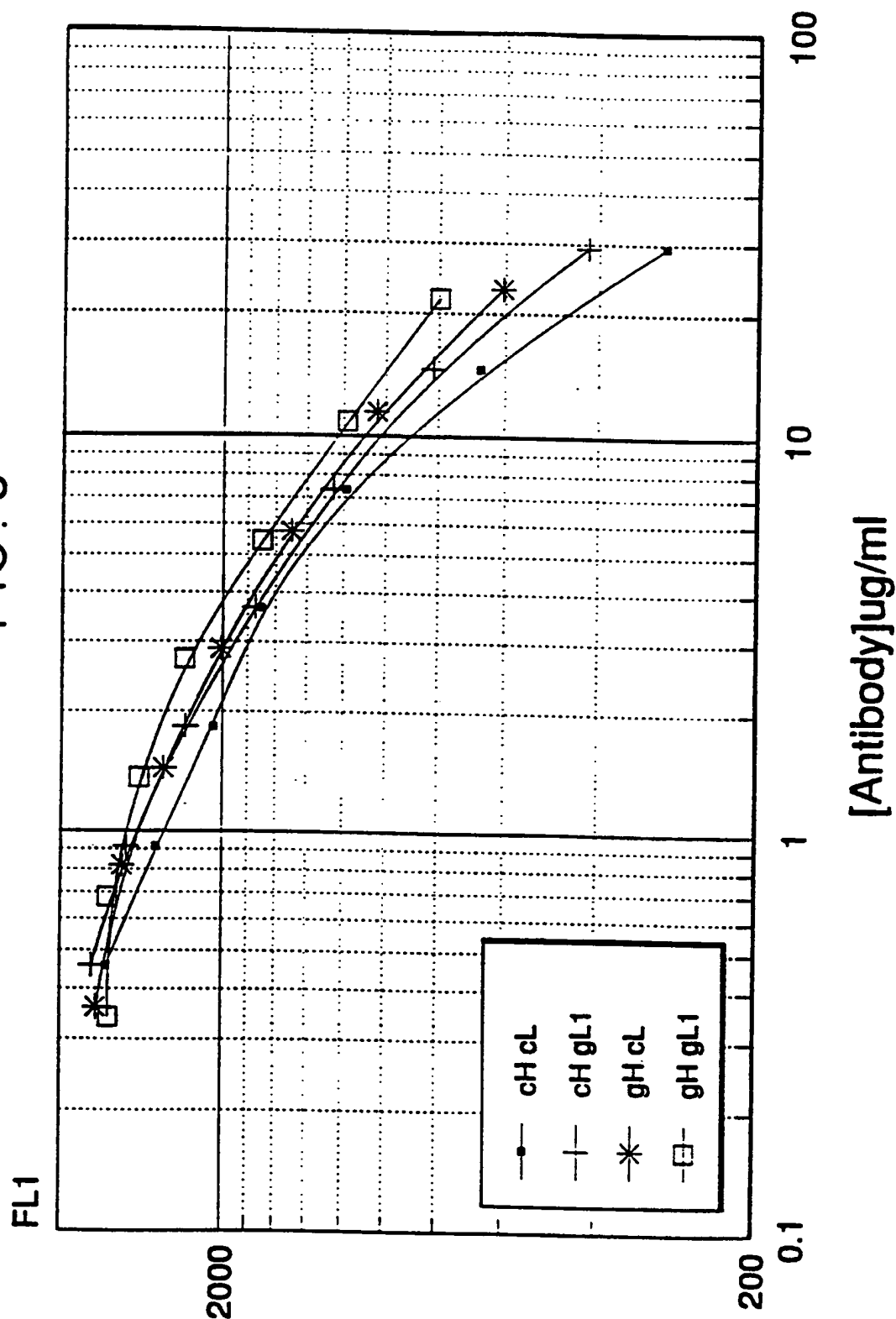
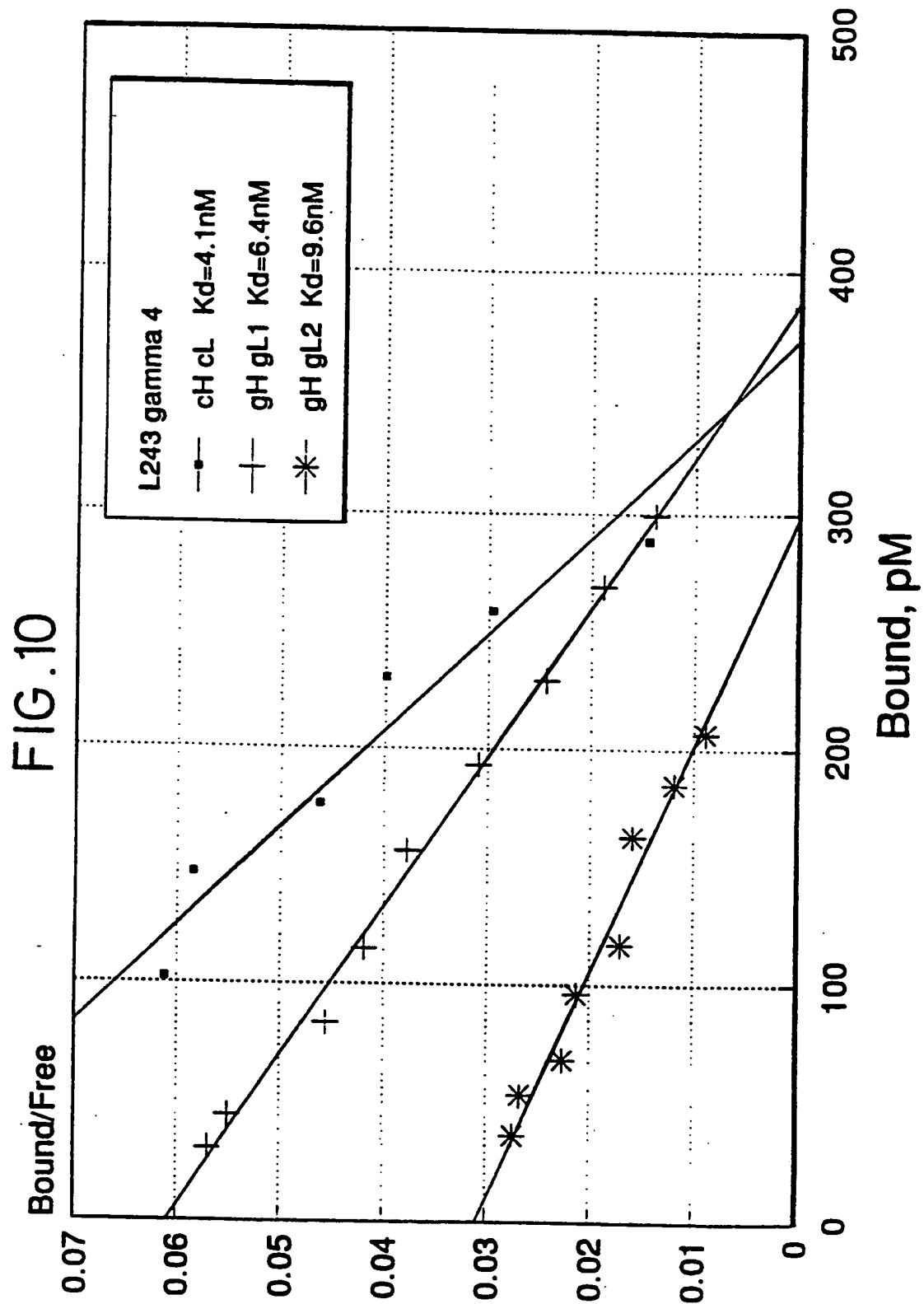


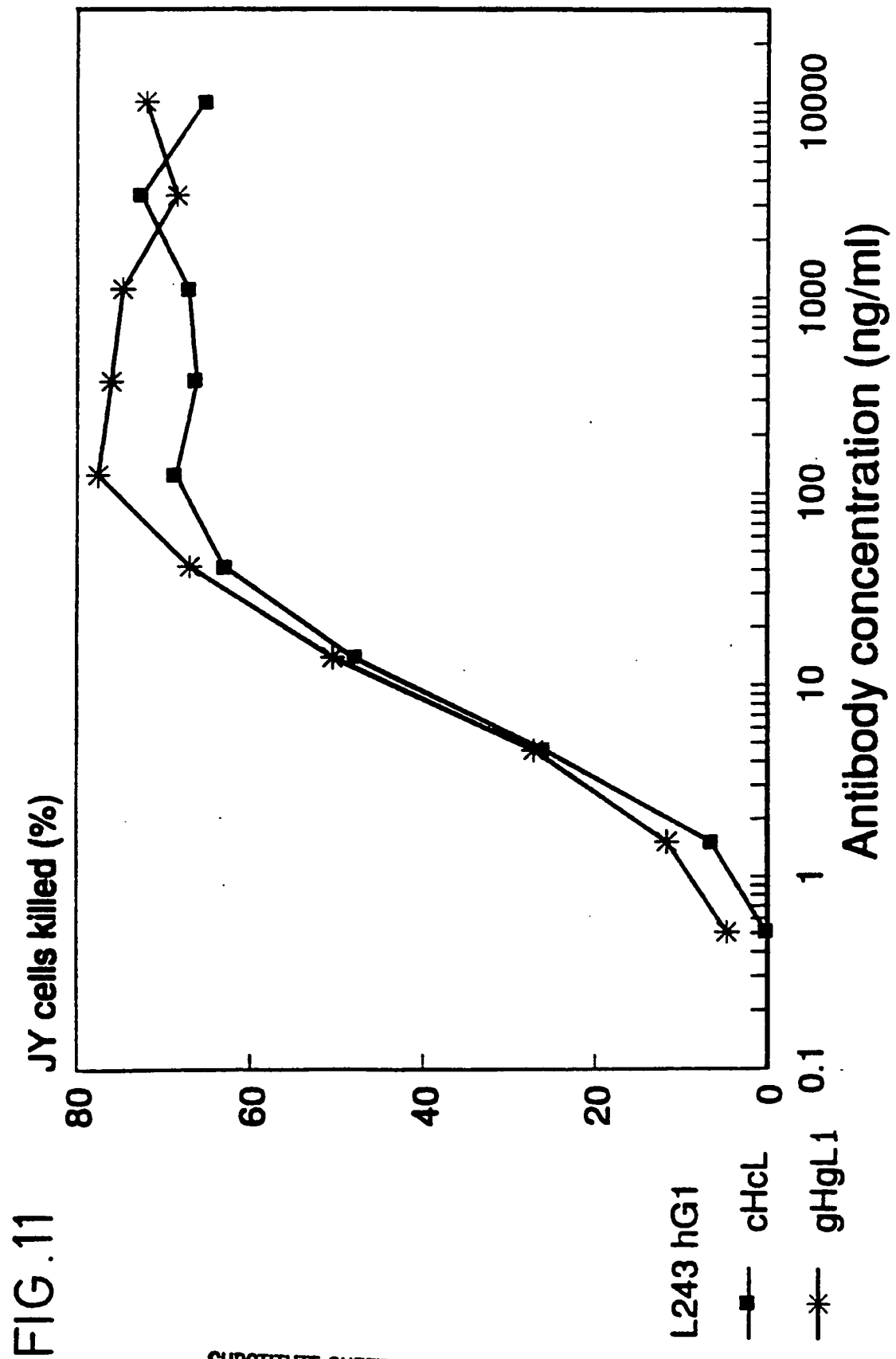
FIG. 9



10 / 20

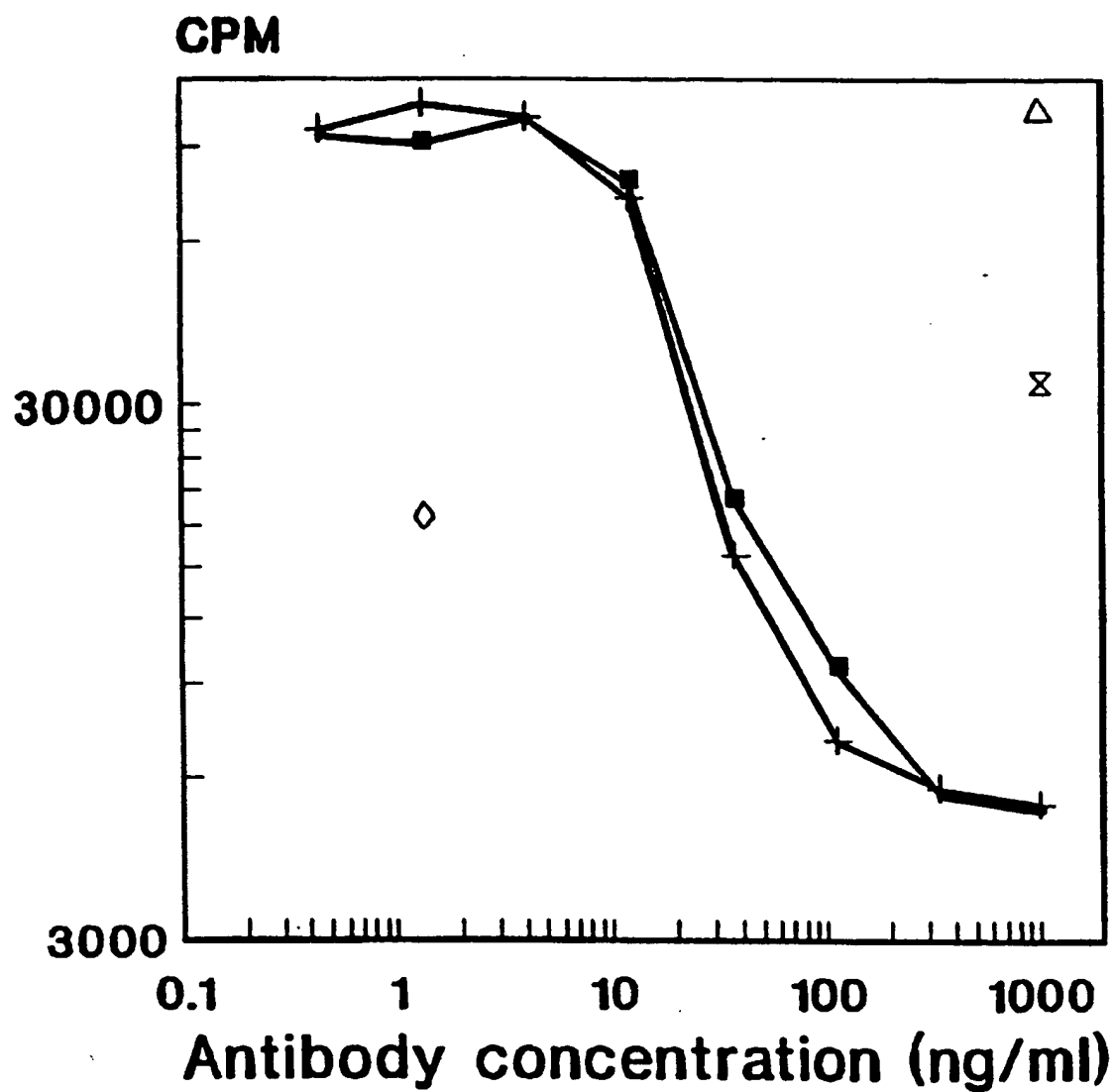


11 / 20



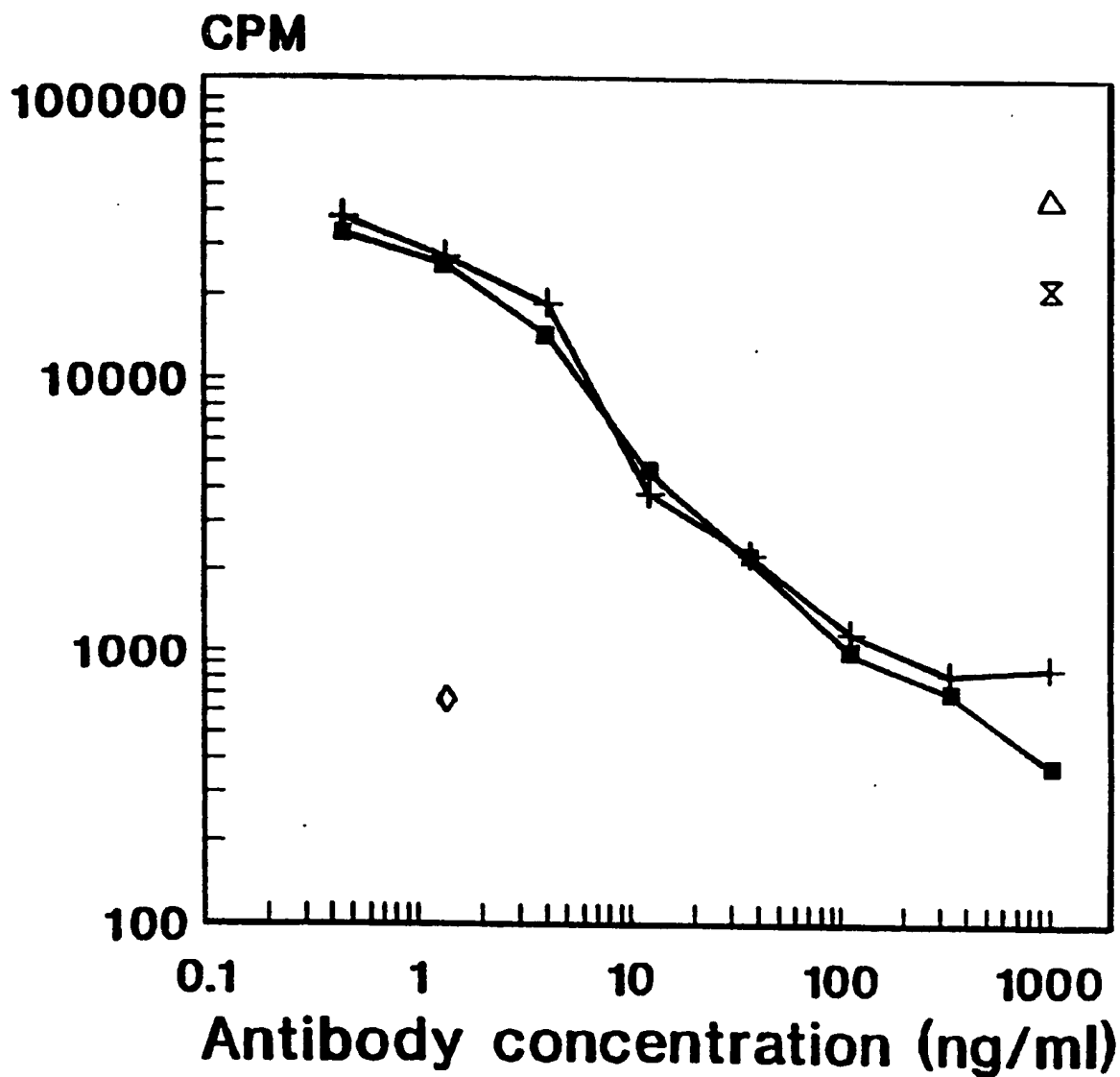
12 / 20

FIG. 12

**L243 Human Isotype**—■— **G1 L243 cHcL**—+— **G1 L243 gHgL1**◇ **Cyclosporin A**△ **M dium control**—x— **R sp nder alone**

13 / 20

FIG. 13



L243 Human Isotype

—■— G1 L243 cHcL

—+— G1 L243 gHgL1

◇ Cycl sp rin A

△ M dium control

⊠ R sponder alone

SUBSTITUTE SHEET (RULE 26)

14 / 20

## FIG. 14

```

      810      820      830      840      850      860
      *      *      *      *      *      *
ACCCC AAAGG CCAAA CTCTC CACTC CCTCA GCTCG GACAC CTTCT CTCCT CCCAG ATCTG
TGGGG TTTCC GGTTT GAGAG GTGAG GGAGT CGAGC CTGTG GAAGA GAGGA GGGTC TAGAC

      870      880      890      900      910      920
      *      *      *      *      *      *
AGTAA CTCCC AATCT TCTCT CTGCA GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA
TCATT GAGGG TTAGA AGAGA GACGT CTC GGG TTT AGA ACA CTG TTT TGA GTG TGT
      E P K S C D K T H T>

      930      940      950      960      970
      *      *      *      *      *
TGC CCA CCG TGC CCA GGTA GGCAG CCCAG GCCTC GCCCT CCAGC TCAAG GCGGG
ACG GGT GGC ACG GGT CCATT CGGTC GGGTC CGGAG CGGGA GGTCG AGTTC CGCCC
      C P P C P>

      980      990      1000      1010      1020      1030
      *      *      *      *      *      *
ACAGG TGCCC TAGAG TAGCC TGCAT CCAGG GACAG GCCCC AGCCG GGTGC TGACA CGTCC
TGTC ACCGG ATCTC ATCGG ACGTA GGTCC CTGTC CGGGG TCGGC CCAGC ACTGT GCAGG

      1040      1050      1060      1070      1080
      *      *      *      *      *
ACCTC CATCT CTTCC TCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC
TGGAG GTAGA GAAGG AGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG AAG GAG
      A P E L L G G P S V F L>

1090      1100      1110      1120      1130      1140
      *      *      *      *      *      *
TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA
AAG GGG GGT TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT
      F P P K P K D T L M I S R T P E V T>

      1150      1160      1170      1180      1190
      *      *      *      *      *
TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC
ACG CAC CAC CAC CTG CAC TCG GTG CTT CTG GGA CTC CAG TTC AAG TTG ACC ATG
      C V V V D V S H E D P E V K F N W Y>

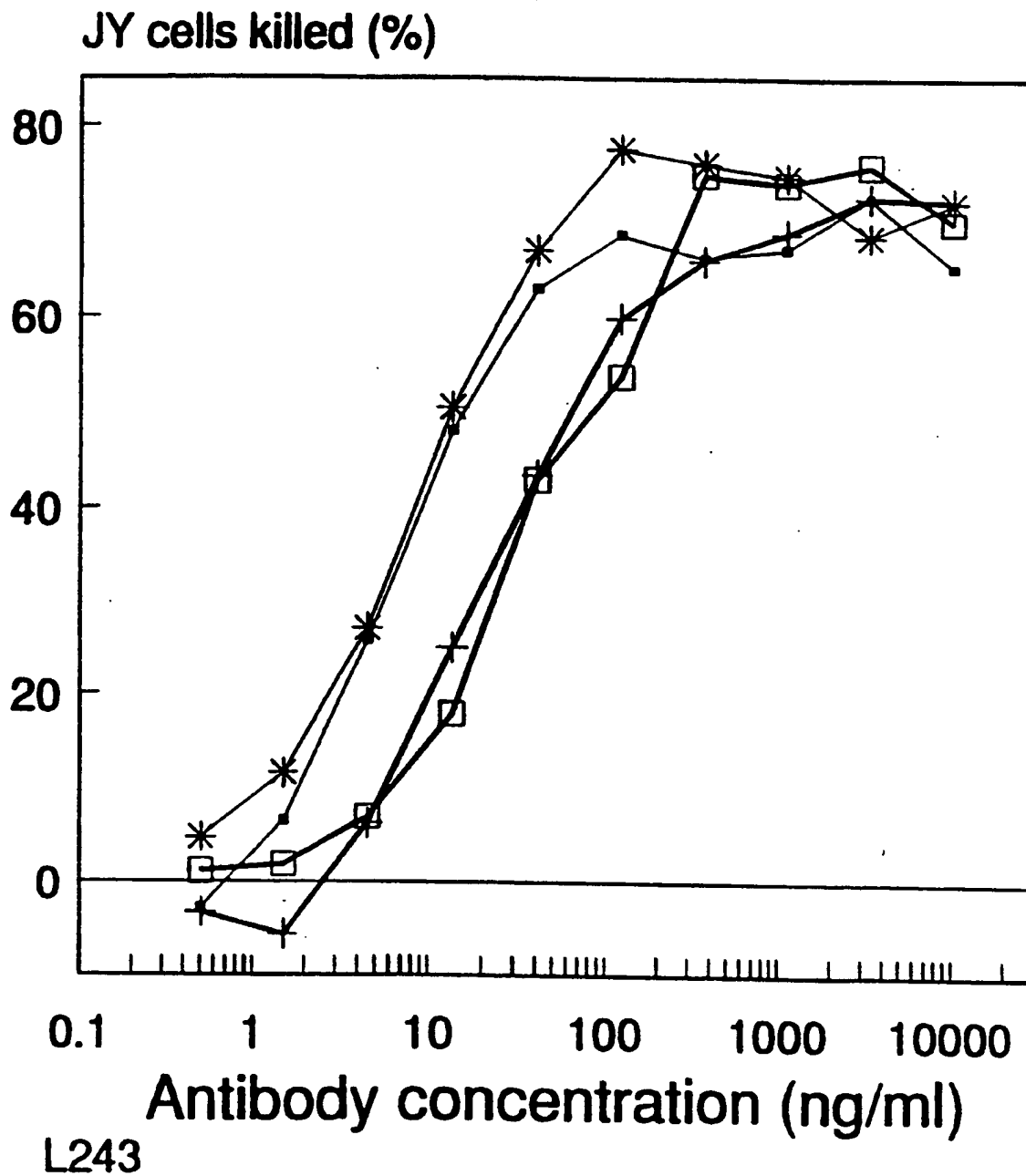
1200      1210      1220      1230      1240      1250
      *      *      *      *      *      *
GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC
CAC CTG CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC ATG
      V D G V E V H N A K T K P R E E Q Y>

```

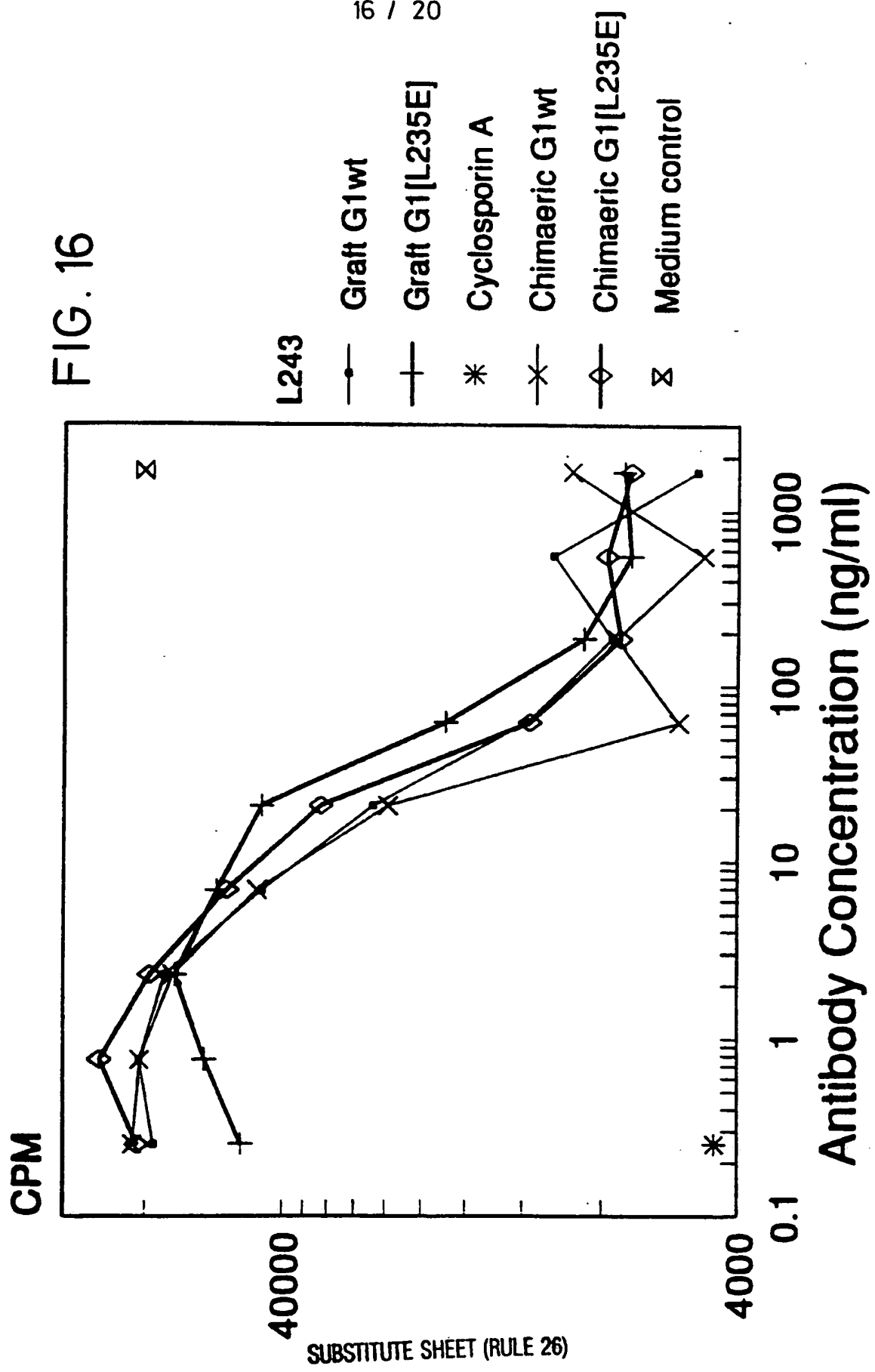


15 / 20

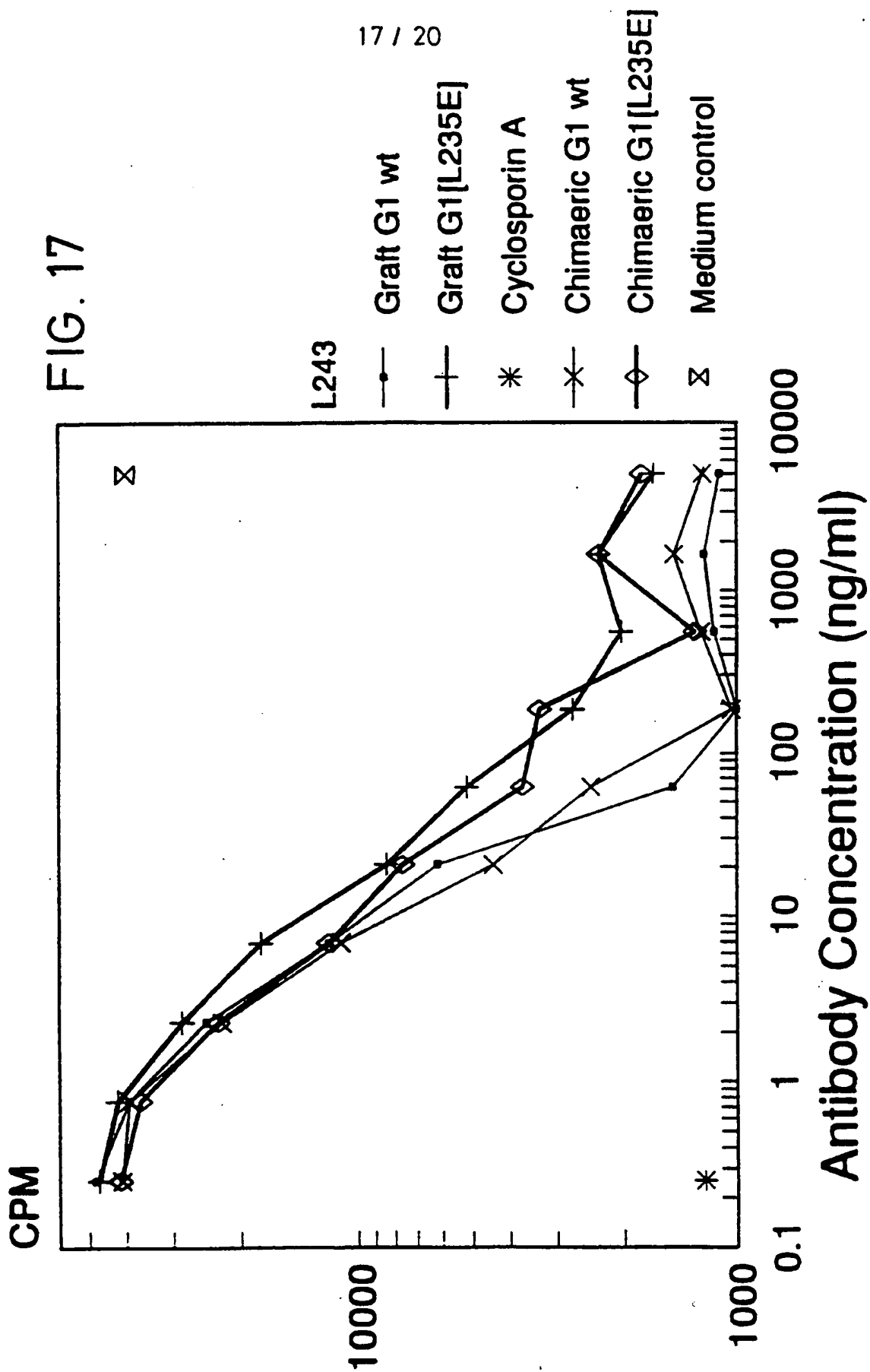
FIG. 15

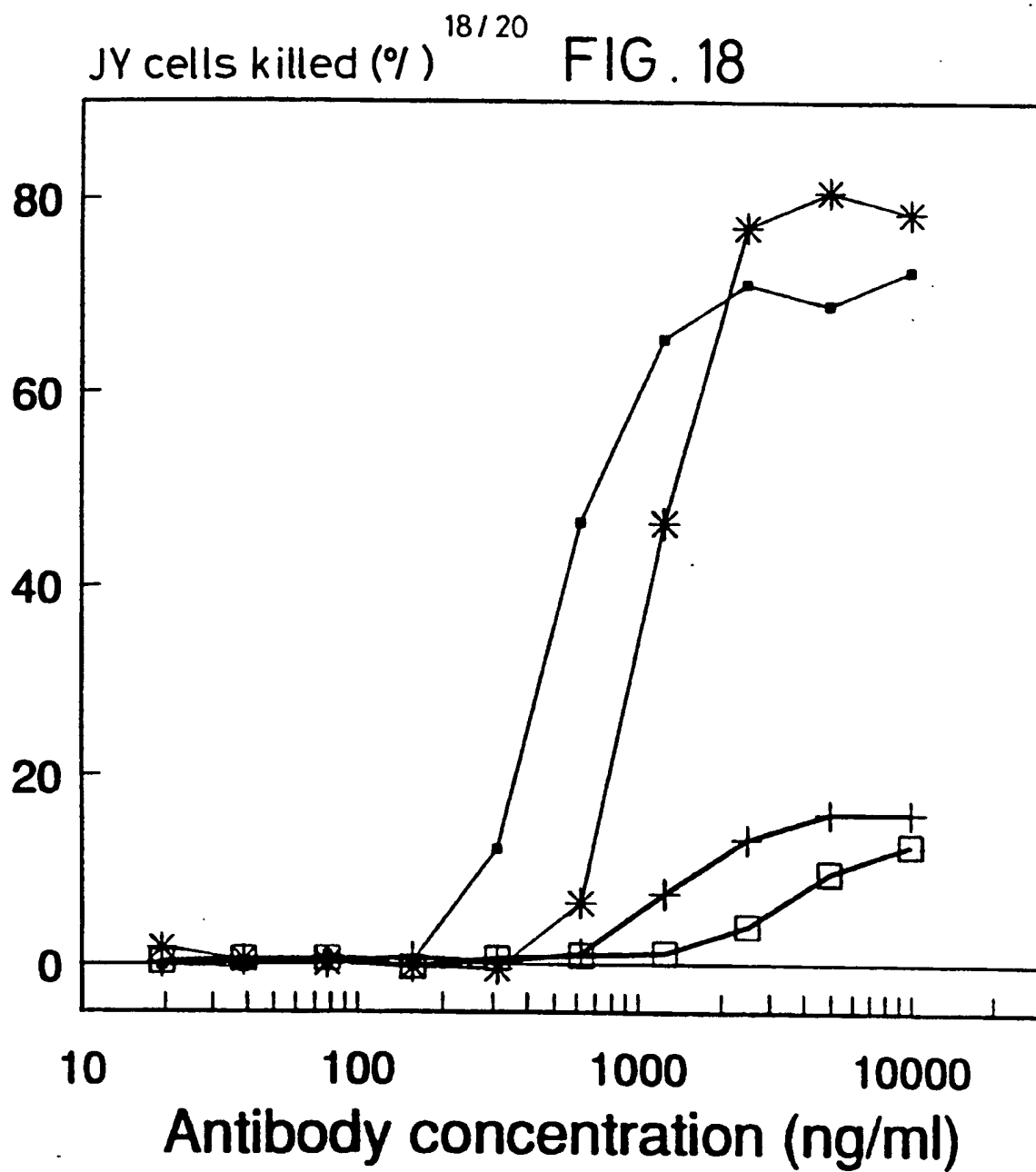


- Chimaeric G1 wt
- +— Chimaeric G1[L235E]
- \*— Graft G1 wt
- Graft G1[L235E]



17 / 20





L243

- Chimaeric G1 wt
- +— Chimaeric G1[L235E]
- \*— Graft G1 wt
- Graft G1[L235E]

## FIG. 19

19 / 20

a. TTC GAA GCC GCC ACC ATG TGG GGA TCT GTT TTC CAT TTT TCA ATT GTA  
 AAG CTT CGG CGG TGG TAC ACC CCT AGA CAA AAG GTA AAA AGT TAA CAT  
                                   M   W   G   S   V   F   H   F   S   I   V>

GAT GCC AGA TGT GAC ATC CAG ATG ACT CAG TCT CCA GCC TCC CTA TCT  
 CTA CGG TCT ACA CTG TAG GTC TAC TGA GTC AGA GGT CGG AGG GAT AGA  
          D   A   R   C   D   I   Q   M   T   Q   S   P   A   S   L   S>

GTA TCT GTG GGA GAA ACT GTC ACC ATC ACA TGT  
 CAT AGA CAC CCT CTT TGA CAG TGG TAG TGT ACA  
          V   S   V   G   E   T   V   T   I   T   C>

b. TTC GAA GCC GCC ACC ATG AGG TGC TCT GCT GAG TTT CTG GGG TTG CTG  
 AAG CTT CGG CGG TGG TAC TCC ACG AGA CGA CTC AAA GAC CCC AAC GAC  
                                   M   R   C   S   A   E   F   L   G   L   L>

CTG CTG TGG CTT ACA GAT GCC AGA TGT GAC ATC CAG ATG ACT CAG TCT  
 GAC GAC ACC GAA TGT CTA CGG TCT ACA CTG TAG GTC TAC TGA GTC AGA  
          L   L   W   L   T   D   A   R   C   D   I   Q   M   T   Q   S>

CCA GCC TCC CTA TCT GTA TCT GTG GGA GAA ACT GTC ACC ATC ACA TGT  
 GGT CGG AGG GAT AGA CAT AGA CAC CCT CTT TGA CAG TGG TAG TGT ACA  
          P   A   S   L   S   V   S   V   G   E   T   V   T   I   T   C>

c. TTC GAA GCC GCC ACC ATG GGC ATC AAG ATG GAG TCA CAG TTC CAG GTC  
 AAG CTT CGG CGG TGG TAC CCG TAG TTC TAC CTC AGT GTC AAG GTC CAG  
                                   M   G   I   K   M   E   S   Q   F   Q   V>

TTC ATA TCC ATA CTG CTC TGG TTA TAT GGA GCT GAT GGG AAC ATT GTA  
 AAG TAT AGG TAT GAC GAG ACC AAT ATA CCT CGA CTA CCC TTG TAA CAT  
          F   I   S   I   L   L   W   L   Y   G   A   D   G   N   I   V>

ATG ACC CAA TCT CCC AAA TCC ATG TCC ATG TCA GTA GGA GAG AGG GTC  
 TAC TGG GTT AGA GGG TTT AGG TAC AGG TAC AGT CAT CCT CTC TCC CAG  
          M   T   Q   S   P   K   S   M   S   M   S   V   G   E   R   V>

ACC TTG ACC TGC AAG GCC AGT GAG  
 TGG AAC TGG ACG TTC CGG TCA CTC  
          T   L   T   C   K   A   S   E>

SUBSTITUTE SHEET (RULE 26)

20 / 20

FIG. 20

